NON-ANTIBIOTIC APPROACHES TO CONTROL PATHOGENS IN THE GASTROINTESTINAL TRACT OF THE BROILER CHICKEN

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ABSTRACT

The purpose of this work was to examine the effectiveness of several replacements for antibiotics in broiler chickens using bacterial challenge models. For this work, pathogen challenge models were developed using three model pathogens; two human pathogens (*Salmonella enteritidis* and *Campylobacter jejuni*), and one poultry pathogen (*Clostridium perfringens*). The first set of experiments involved the selection and use of 2 model probiotics; *Bifidobacterium animalis* and *Lactobacillus fermentum*. Oral administration of either probiotic did not significantly reduce ($P < 0.05$) the level of intestinal colonization by either *S. enteritidis* or *C. jejuni* in experimentally infected broiler chickens. The next set of experiments examined the effectiveness of orally administered, pathogen-specific antibodies obtained from hyperimmunizing laying hens in controlling bacterial infections with *S. enteritidis*, *C. jejuni* or *Clostridium perfringens* in broiler chickens. Regardless of the concentration, or mode of administration, anti-*S. enteritidis* hen-egg antibodies or anti-*C. jejuni* hen-egg antibodies were unable to significantly reduce ($P < 0.05$) the intestinal colonization by either pathogen in experimentally infected broiler chickens. Likewise, administration of anti-*C. perfringens* hen-egg antibodies did not reduce intestinal colonization by *C. perfringens*, and actually exacerbated the clinical outcome of this important poultry pathogen by significantly increasing ($P < 0.05$) intestinal lesions scores compared to negative control birds. Lastly, the effect of dietary protein source on intestinal *C. perfringens* populations was investigated. In broiler chickens experimentally infected with *C. perfringens* and fed diets which varied in the source of dietary protein, it was shown that birds fed fish meal, meat/bone meal, feather meal and potato protein

concentrate had significantly higher intestinal *C. perfringens* counts than the birds fed corn gluten meal, soy or pea protein concentrates or the control diet ($P < 0.05$). Further, it was shown that the glycine content of the diets and ileal contents was significantly, positively correlated with *C. perfringens* numbers in ileum and cecum. It is concluded that although the intervention strategies employed in these studies show promise, diet composition clearly had the largest effect on intestinal bacterial populations. Further studies are required to examine both the impact that diet and these intervention strategies have on the factors which control intestinal colonization by pathogens on a case by case basis.

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LIST OF ABBREVIATIONS

AGP	antibiotic growth promoter
FM	fish meal
MBM	meat and bone meal
FeM	feather meal
PoPC	potato protein concentrate
PePC	pea protein concentrate
SPC	soy protein concentrate
CGM	corn gluten meal
CFU	colony forming units
LAB	lactic acid bacteria
BHI	brain heart infusion
SEM	standard error of the mean
PBS	phosphate-buffered saline
HEA	hen-egg antibody
OD	optical density
SE	standard error
ABTS	2,2’-azino-bis-(3-benzthiazoline-6-sulfonic acid

1. INTRODUCTION

Although it was Pasteur who postulated that microorganisms are necessary for normal life, it has only been in the past several decades that the microflora of the gastrointestinal tract has generated much interest amongst researchers. With the exception of pathogens and the diseases that they cause, there was little appreciation of the normal intestinal microflora. In recent years however, there has been an increase in our understanding of the gastrointestinal tract, the microflora contained therein, and how management practices can influence both. Recent research is revealing that the gastrointestinal tract is a much more complex ecosystem than was previously thought, and one in which the host and the microflora are dependant upon each other for health and survival.

Historically, the intestine has been viewed as a black box with inputs and outputs the only parameters that were considered. The introduction of in-feed antibiotics in the 1950s effectively controlled disease and improved animal performance (Dibner and Richards, 2005; Jones and Ricke, 2003), however the effectiveness of antibiotics also reduced the importance of understanding gastrointestinal microbiology. As a result, there is a huge gap in our understanding of the gastrointestinal microflora and its effects on the host at the most basic level. Current research however, has demonstrated the

important role that the normal gastrointestinal microflora plays in animal health and nutrition, as well as highlighting the ecological significance of intestinal pathogens.

According to the Chicken Farmers of Canada (2004), chicken consumption is increasing both nationally and provincially in Saskatchewan. In terms of consumption, chicken is second only to beef and in 2003 an average of 30.5kgs of chicken per person was consumed. In this same year, Saskatchewan had 97 chicken producers which generated 30,456,000 kgs of meat, with farm receipts totaling $50.5 million dollars. Clearly, the production of chicken in Saskatchewan represents a significant contribution to the economy which is expected to not only continue, but increase as well. Since 2000 there has been a 24.7 % increase in production and it is projected to increase between 2.0 and 2.5% annually in the future. Thus, the future profitability of the poultry industry in Saskatchewan and elsewhere depends upon not merely maintaining current production levels, but increasing them with to meet increasing demand. In addition to maintaining production however, profitability also requires that poultry products are safe and free from the microorganisms that cause food-borne illnesses. Consumer awareness, coupled with high profile food-borne disease outbreaks, have eroded consumer confidence in the meat and poultry supply and consequently the presence of bacterial pathogens in the food supply is a major concern to both the consumer and regulatory agencies. Thus, an improved understanding of the microbial ecology of the gastrointestinal tract, including methods to manage it, is important to the sustainability of the poultry industry in Saskatchewan and around the world in the prevention of disease and foodborne illness and the enhancement of the growth performance of broiler chickens.

2. LITERATURE REVIEW

2.1. The gastrointestinal microflora

The gastrointestinal tract of monogastric animals harbors a large collection of different microbial species. The number of species in humans is generally estimated to be between 400 and 500, although some researchers have suggested that this may represent only 10% of the total number of species present (Berg, 1996; Finegold *et al*., 1975; Savage, 1977). Recent research has suggested that the chicken gastrointestinal microflora may contain more that 650 species of which more than half are from previously unknown bacterial genera (Apajalahti *et al.*, 2004). The number, type, and distribution of microorganisms that comprise the normal flora differ between animal species, however, in general there is a population gradient from the proximal to distal ends of the gut (Engberg *et al.*, 2000), where overall anaerobic and facultatively anaerobic gram positive microorganisms appear to dominate (Mackie *et al*., 1999; Savage, 1977). Even the stomach, which was once thought to be free of indigenous microflora, is home to microorganisms albeit in low numbers compared to the colon (Lee *et al.*, 1993). Like the stomach, the proximal two-thirds of the small intestine was once thought to be free of a resident microflora. The cultured microbes were thought to be transient, presumably associated with food as it passed through the intestine. It has been shown, however, that the mucosal epithelium of the duodenum and jejunum can harbor microflora under certain circumstances, and may in fact be permanently

colonized by bacteria (Ciampolini *et al*., 1996; Savage, 1986). However, it is the distal one-third of the small intestine and the large intestine which constitute the bulk of the intestinal microflora. The total microbial count of the gastrointestinal tract is estimated to be over 10^{14} microorganisms and thus outnumbers the total 10^{13} cells in the adult human body (Savage, 1977).

2.2. Acquisition and succession

The microflora begins to colonize the gastrointestinal tract of young animals immediately at birth or hatch, and the interaction between host and microflora continues throughout life. In well recognized and characterized patterns of succession, the microflora develops in response to exposure, as well as host health and nutrition (Amit-Romach *et al*., 2004; Lu *et al*., 2003; Mackie *et al*., 1999; Simpson *et al*., 1999; van der Wielen *et al*., 2002b). These patterns of succession have been found to be similar in chicks, piglets, calves, and humans (Mackie *et al.*, 1999). Sterile at hatch or birth, the complete microflora or climax community develops slowly and depending on the animal species, may take up to 2 years to fully develop as is the case with humans (Falk *et al.*, 1998). Bacteria of the gastrointestinal tract for the most part derive their energy from dietary components consumed by the host. These dietary components are either resistant to the host's digestive enzymes or slowly digested, and as a result the composition and structure of the diet is largely responsible for the microbial community of this ecosystem (Apajalahti *et al.*, 2004). The impact of diet may be readily be observed in community structure and diversity (Apajalahti *et al*., 2001; Gibson *et al*., 1996; Reid and Hillman, 1999).

Recent research by Apajalahti *et al*. (2004) has shown that within 24h post-hatch ileal and cecal bacterial numbers in chickens can be as high as 10^8 to 10^{10} per gram of digesta, respectively. During the subsequent 3 days these numbers have been observed to increase to between 10^9-10^{11} and remain relatively stable for the following 30 days. The authors also suggested that although the numbers remain relatively stable, the composition of the microflora undergoes major changes over the same time period. The total number of gut bacteria observed in chickens is consistent with the bacterial numbers found during the same time period in other animal species (Snel *et al.*, 2002). Using molecular methods to evaluate the composition of the gastrointestinal microflora, Lu *et al.* (2003) demonstrated that community stability based on community profiles obtained from 16s gene sequences could be directly related to the age of the bird. During the first week of life it has been shown that enterococci and lactobacilli dominate in the crop, duodenum and ileum while it has been shown that in the cecum it is coliforms, enterococci and lactocbacilli that are the predominant microorganisms (Barnes, 1972; Mead and Adams, 1975; van der Wielen *et al*., 2000). Initially at low numbers, after only 5 to 6 hours there may be 10^9-10^{10} CFU/g of feces in chicks (Snel *et al.*, 2002).

Many of the initial microorganisms found in the gastrointestinal tract are transient and are not able to actually colonize the gastrointinal tract (Barnes *et al.*, 1980). However, both colonizing and transient microorganisms change the physicochemical properties of the intestinal environment which results in a suitable environment for the succession of subsequent microbial species which ultimately comprise the climax community of mature animals. The observed changes occur via the production of

various fermentation products such as volatile fatty acids (VFAs) (van der Wielen *et al.*, 2000), which inhibit some species of gastrointestinal microorganisms, and a more reduced intestinal environment which is necessary for the obligate anaerobes which dominate the intestinal microflora of mature animals particularly in the cecae (Lan *et al*., 2002; Apajalahti *et al*., 2004).

2.3. Climax community

Over time, a stage in community succession is achieved whereby the community has become relatively stable through successful adjustment to its environment. This stability is termed a "climax community". The climax community is similar in various animal species, however considerable variation does occur (Zhu *et al.*, 2002), and although the climax community is composed of predominantly anaerobic gram positive species (Barnes *et al*., 1972; Lu *et al*., 2003), research has shown that the presence of facultative anaerobes or aerobes is needed for optimum intestinal health and disease resistance (Goren *et al.*, 1984). The factors which contribute to the development and maintenance of the normal climax community of adult chickens include environment, host-specific determinants, and the intestinal compartment in which they are found (Lu *et al*., 2003; van der Wielen *et al*., 2002b).

The gastrointestinal microflora of older birds, and thus the climax community, has been shown to be more diverse than in younger birds using either traditional (Barnes *et al*., 1972; van der Wielen *et al*., 2000) or molecular methods (van der Wielen *et al.*, 2002b) for the identification of gastrointestinal microflora. This is particularly evident in the cecum where there appears to be a much more complex microbial community,

compared to the small intestine and crop where only a few species appear to dominate (Lu *et al*., 2003; van der Wielen *et al*., 2002b).

The climax community found in healthy animals serves to protect the animal from the colonization by pathogenic species (Mackie *et al*., 1999; Savage, 1977). Interestingly, despite the recognition that a completely developed microbial community is required for optimal health, and that the time required to reach adulthood by chickens is approximately 30 weeks, broiler chickens are marketed well before this age. It is important, therefore, to bear in mind that the microflora of a bird this age, although relatively stable, has not yet fully developed (Lu *et al.*, 2003).

2.4. Inter-species differences in intestinal microflora

Compared to many other animal species, the chicken has a relatively simple modified gastric system. Perhaps the most significant difference between modern poultry and mammalian agricultural species is the absence of a weaning period and complete separation of hen and chick before hatch. This separation leads to an artificial pattern of succession in terms of the colonizing species, and ultimately delays the development of the climax community (Snel *et al.*, 2002). In addition to rearing practices, however, the physiological and anatomical differences between chickens and other monogastric animals result in additional differences in their gastrointestinal microflora (Hooper and Gordon, 2001; Lu *et al*., 2003). As well as species-specific differences, research using molecular methods for the enumeration and identification of gastrointestinal microflora has shown that differences between individuals can also be observed (van der Weilen *et al.*, 2002). This suggests that there exist host-specific factors which are important for the establishment of the gastrointestinal microflora, and

these factors potentially lead to the development of a unique gastrointestinal microbial community in chickens (Lu *et al*., 2003; Tannock *et al*., 1982).

2.5. Spatial distribution of chicken gastrointestinal microflora

Virtually all the studies where traditional bacterial culture methods have been used consistently report that there are 10^{11} to 10^{12} bacteria per gram of intestinal contents in the lumen of an adult broiler chicken (Fuller, 1992). Within the small intestine the observed intestinal microflora appears to be established and stable after 2 to 3 weeks post-hatch (Lu *et al*., 2003; Snel *et al*., 2002), whereas in the more microbially complex cecum stability is not achieved until much later at 6 to 7 weeks (Coloe *et al.*, 1984), and may even take as long as 30 weeks (Lu *et al.*, 2003). As with all animal species, there are spatial differences within the gastrointestinal microflora of chickens. With the exception of the right and left cecae, each compartment of the gastrointestinal tract has been shown to have its' own specific microbial community (van der Weilen *et al.*, 2002). These differences are both in the number of microorganisms found, but also in the type. Some of these major differences are outlined below.

2.5.1. Crop

The crop serves as a storage organ which partially regulates the entry of the ingested food into the gizzard. This allows the chicken to eat its daily ration in a short period and digest it later. Considerable microbial growth occurs in the crop which might contribute to feed digestion and is therefore beneficial to the bird (Champ *et al.*, 1983). The time feed is in the crop depends on a number of factors including the amount, consistency, moisture content, and access to feed. These factors also influence the microbial growth found in this organ. Some of the bacterial species isolated from the crop of chickens

include *Escherichia coli,* enterococci, staphylococci, lactobacilli, *Campylobacter* spp., and *Salmonella* spp. (Frei *et al.*, 2001), however many of these are transient species and after the first week the dominant species found in the crop is *Lactobacilli* (Barnes *et al*., 1972; Mead, 1997; Mead and Adams, 1975; van der Wielen *et al*., 2002b; van der Wielen *et al*., 2000). On the epithelial surface of the crop the *Lactobaccillus* spp. form a layer of cells up to 3 deep which restricts the available crop epithelium surface for colonization by pathogenic species (Fuller, 1973). Shortly after feeding, the pH of the crop decreases to approximately 5.0 due to the bacterial production of lactic acid. This reduced pH may contribute to digestion via the hydrolysis of stored feed, but also may have a bacteriostatic or bacteriocidal activity against bacteria sensitive to this pH, and consequently may protect the chicken from ingested pathogenic bacteria (Fuller, 1977; Mead, 1997). Thus, the crop also has an influence the microbial ecology of the entire gastrointestinal tract (Maisonnier *et al.*, 2003).

2.5.2. Proventriculus (stomach) and ventriculus (gizzard)

The proventriculus is a glandular organ which corresponds to the stomach of mammals. It produces a gastric juice containing hydrochloric acid and proteolytic enzymes. However, it differs from the mammalian stomach in that little mixing or holding of food occurs in it. From the proventriculus, the food moves to the ventriculus (or gizzard), a muscular organ where the food is ground and mixed with the gastric juice. It has been shown that similar to the crop, the low pH of the gizzard does define the microbial population in distal portions of the gastrointestinal tract (Bjerrum *et al.*, 2005). In terms of the microbial population that it harbors, a search of the literature shows little is known. The harsh environmental conditions found in these two regions of

the gastrointestinal tract would suggest that the vast majority of the culturable microbes found are transient, however the discovery of resident microbes in the stomach of humans (Lee *et al.*, 1993) indicates that these regions of the chicken intestine may also have a resident population of microorganisms with an as of yet unknown role in the microbial ecology of the gastrointestinal tract.

2.5.3. Small intestine

In the small intestine, the majority of the ingested nutrients are broken down, digested, and adsorbed by the host. Thus in terms of microbial nutrition, this is an environment with intense competition for available nutrients. As in the crop, *Lactobacillus* spp. appear to dominate in the small intestine (Barnes *et al*., 1972; Mead, 1997; Mead, 1997; van der Weilen *et al*., 2002). Using molecular techniques, Lu *et al.* (2003) found that out of a total number of 614 16s rDNA sequences present, *Lactobacillus* spp. comprised 68.5% of the total number. The remainder of the ileal community was composed of smaller numbers of other species, including members from 14 other genera. These researchers also demonstrated population shifts within the ileal community over time. This age-dependant development has been observed by others (van der Wielen *et al.*, 2002b), and has been shown to be unique to this region of the gastrointestinal tract.

2.5.4. Cecum

The cecae of adult chickens provides a relatively stable environment and as such harbors the largest and most complex microbial community within the gastrointestinal tract (Mead, 1997). As well as being the largest assemblage of gastrointestinal microbes, it is also the most varied (Apajalahti *et al*., 2001; Mead and Adams, 1975;

van der Weilen *et al*., 2002; Zhu *et al*., 2002). As with all regions of the gastrointestinal tract, there are age-dependant differences in the microbial community within the cecum (Amit-Romach *et al*., 2004; Lu *et al*., 2003). At 1 day of age the number of bacteria found in cecal contents is as high as 10^6 CFU/mL (van der Wielen *et al.*, 2000). The population is composed of predominantly *Lactobacillus* spp. and is not significantly different from the small intestine (Lu *et al.*, 2003). As the chicken ages, however, the *Lactobacillus* spp. become less dominant as both the number and diversity of the cecal microflora increase (Barnes *et al*., 1972; Lu *et al*., 2003; Mead and Adams, 1975; van der Wielen *et al*., 2002b). The increase can take up to 30 days or longer to develop (Coloe *et al.*, 1984; Lu *et al.*, 2003). These changes make the microbial ecology of the cecae much different compared to other parts of the gastrointestinal tract (Lu *et al.*, 2003), but as with other regions of the gastrointestinal tract, they are in response to the available nutrients and environmental conditions. Lu *et al.* (2003) were able to determine that regardless of age, species from the genera *Clostridium, Eubacterium,* and *Ruminococcus* dominated the microbial community of the cecum.

2.6. Importance of gastrointestinal microflora in health and disease

Work with germ-free animals has shown that the gastrointestinal microflora has an enormous amount of influence on the development of the chicken gastrointestinal tract (Furuse and Okumura, 1994). The observed effects include an influence on the gross physiology of the intestine, but also immunological, nutritional and protective influences as well. The host microflora also provides a number of nutritional compounds, which have a profound effect on the overall health and performance of the host animal. Clearly, the effects are beneficial; however, the benefits are offset to a

certain degree by a physiological cost to the host animal. The commensal microflora competes with the host for ingested nutrients, secretes toxic compounds, and stimulates a continuous immune response. Thus, the challenge for gastrointestinal microbiologists and animal scientists is to understand the microbial ecology and microbiological processes of the gastrointestinal tract in such as way as to maximize the benefits, while minimizing the cost of maintaining what is essentially another organ.

2.6.1. Benefits

Perhaps the most beneficial effect of the gastrointestinal microflora is the resistance to colonization by pathogenic microorganisms it provides. This phenomenon has been termed as bacterial resistance, colonization resistance, or competitive exclusion. Although the exact mechanisms by which this protection is conferred are unknown, it is hypothesized to be due to the production of antimicrobial compounds such as organic acids and bacteriocins, stimulation of the immune system, or competition for nutrients and specific epithelial binding sites (Nurmi *et al*., 1992; Rolfe, 1997). Experimental evidence to support this hypothesis can be demonstrated using germ-free chickens which are much more susceptible to colonization by pathogens than conventional animals (Fukata *et al*., 1991; Hudault *et al*., 1985).

The gastrointestinal microflora also produce a number of nutrients that become available to the host animal, including volatile fatty acids (VFAs), amino acids, B vitamins and vitamin K (Savage, 1986; Snel *et al*., 2002; Wostmann, 1996). VFAs such as lactate, acetate, propionate, and butyrate produced from fermentable carbohydrates (Barnes, 1979; van der Wielen *et al.*, 2000) are absorbed by the gut and serve as an energy source for the gut itself as well as the body as a whole (Hegde *et al.*, 1982). In

addition to being a source of energy, the volatile fatty acids have been shown to stimulate the growth of intestinal epithelial cells, which leads to increased villus size and intestinal absorptive capacity (Cook and Bird, 1973; Tellez *et al*., 1993). Additionally, they also contribute to determining the microorganisms present in the distal portion of the gastrointestinal tract via bacterial inhibition and immunomodulation of the host (Hara *et al*., 2003; van der Wielen *et al*., 2000). The role that microbially derived amino acids and vitamins may contribute to chicken health using modern rearing practices is unclear (Dibner and Richards, 2005).

As well as providing colonization resistance and the production of nutrients, the normal microflora also benefit the host by stimulating both specific and non-specific host defenses which serve to protect the host from not only invading pathogenic microorganisms, but also the inappropriate growth of normal gastrointestinal microorganisms. These host defenses include production of the mucus layer which overlies the gastrointestinal epithelium and acts as a barrier to the epithelium itself (Deplancke and Gaskins, 2001; Sharma and Schumacher, 1995), as well as both specific and non-specific immunity (Jeurissen *et al.*, 2002). This constant stimulation of the immune system has physiological cost to the host. It has been estimated that in pigs, for example, the production and secretion of protective IgA amounts to several hundred grams of protein during the production cycle of a market pig (Gaskins, 2001).

2.6.2. Costs

In addition to the metabolic costs associated with immunostimulation and the above described production and secretion of immunoglobulin, even under ideal conditions there are additional metabolic costs associated with the gastrointestinal microflora.

Perhaps most importantly, is the direct competition for nutrients ingested by the host. It has been estimated that the microflora may consume 10 to 20% of the ingested carbohydrates and protein that would otherwise be available to the host (Apajalahti *et al.*, 2004).

The resident microflora also decrease fat digestibility through the catabolism of bile (Knarreborg *et al.*, 2004), especially by *Lactobacillus* spp. (Baron and Hylemon, 1997). The deconjugation of bile by the gastrointestinal microflora thus impairs lipid absorption and may also produce a number of anti-nutritional catabolites from the degradation of bile (Baron and Hylemon, 1997).

Additional toxic catabolites are produced by the gastrointestinal microflora through the fermentation of amino acids (Gaskins, 2001). These toxic catabolites contribute to increased epithelial cell turnover rates, as well as contribute to diarrhea in the host animal (Gaskins, 2001). Not only does the gastrointestinal microflora increase epithelial cell turnover rates due to the production of toxic catabolites, they also shorten the lifespan of absorptive enterocytes and mucus secreting goblet cells (Gaskins, 2001).

2.7. The role of normal microflora in disease prevention

Despite the metabolic costs, however, there appears be an advantage to the presence of the gastrointestinal microflora, and for the most part a symbiotic relationship has developed over time (Farthing, 2004). The indigenous or commensal microorganisms benefit from the protection and various niches that the gastrointestinal tract provides, and the host benefits from the presence of the microflora. Perhaps the most important benefit of the normal microflora is in the prevention of disease. The presence of a fully developed and healthy gut microflora serves to reduce the chance of intestinal

colonization by non-indigenous microflora especially pathogens (Snel *et al*., 2002; van der Waaij *et al*., 1971). This has been termed colonization resistance and is the basis for many probiotic strategies. The inability of pathogenic microorganisms to attach and colonize the epithelial surface also reduces their translocation across this important barrier and their ability to cause disease in the animal host (Wells, 1990). Research using germ-free animals has shown that in the absence of intestinal microflora, animals are much more susceptible to pathogen colonization than conventional animals (Harp *et al*., 1992; Srivastava, 1978; Wells *et al*., 1988; Wells *et al*., 1982), however the exact mechanisms by which the normal intestinal microflora impede colonization by pathogens is unclear. Proposed mechanisms include colonization resistance (Corrier *et al*., 1991; Gorbach *et al*., 1988), secretion of antimicrobial compounds such as volatile fatty acids (van der Wielen *et al.*, 2002a) or bacteriocins (Gusils *et al.*, 1999; Portrait *et al.*, 1999), and direct stimulation of immune system (Jeurissen *et al.*, 2002).

2.8. Normal microflora as the cause of disease

The normal microflora contains many microorganisms that can be pathogenic to the host under certain conditions. These opportunistic pathogens are always present, but only cause disease when the finely balanced intestinal ecosystem is perturbed. Under these conditions the microorganism can proliferate in the gastrointestinal tract and subsequently translocate across the epithelial barrier resulting in disease or death of the host (Farthing, 2004). This can occur when there is intestinal overgrowth of a commensal microorganism in the gastrointestinal ecosystem (Fukata *et al.*, 1991), defective barrier function (Farthing, 2004), or a compromised immune status of the host (Shanahan, 2002). Oftentimes these conditions result from a primary infection due to a

pathogenic bacteria or virus (Bano *et al*., 2003; Glisson, 1998; Pakpinyo *et al*., 2003). In addition to opportunistic pathogens, many commensal microorganisms have the potential to become pathogenic. Transfer of genetic material encoding one or more of a series of virulence factors has been shown to transform a commensal microorganism to a pathogenic microorganism (Ewers *et al.*, 2004). Thus, the relationship that the host has with the intestinal microflora, although beneficial, is not without a certain degree of risk.

2.9. Bacterial pathogens and their effect on poultry production

Even in the absence of disease, both opportunistic and overtly pathogenic microorganisms can be isolated from the gastrointestinal tract of chickens. The presence of these microorganisms is ultimately the cause of gastrointestinal infections in poultry which reduce intestinal health and contribute to reduced flock performance. In addition to the pathogens which cause disease in poultry, poultry may act as a reservoir of human pathogens (Mead *et al.*, 1999). Control strategies for the reduction in enteric diseases are almost exclusively delivered via the feed, however the overall low incidence of enteric diseases in modern production systems is also a function of improvements in general poultry husbandry, nutrition, feed quality and veterinary surveillance programs (Dekich, 1998). In terms of the loss to producers annually, it is difficult to estimate, however any strategies which reduce the incidence of disease in poultry are clearly important to all facets of the industry. To date, preventative medicine has been the most successful strategy used to date, including the use of preventative feed medication or antibiotic growth promoters (Dekich, 1998).

2.9.1. Poultry pathogens

A number of enteric bacterial disease are recognized in poultry, some of which are primarily limited to the gastrointestinal tract, whereas others move beyond the confines of the gastrointestinal tract and affect a number of organs (Porter, 1998). Common enteric diseases affecting chickens and the bacterial pathogens that cause them include; necrotic enteritis caused by *Clostridium perfringens* Type A or C (Al-Sheikhly and Truscott, 1977), colibacillosis caused by *E. coli* as either a primary or secondary pathogen (Dho-Moulin and Fairbrother, 1999), fowl cholera caused by *Pasteurella multocida* (Rhoades and Rimler, 1989), salmonellosis caused by number of *Salmonella* spp. including *pullorum* and *gallinarum* (Porter, 1998), and spirochetosis caused by at least 2 species of *Brachyspira,* formerly known as *Serpulina* (Stephens and Hampson, 2001).

2.9.2. Human foodborne pathogens associated with poultry

United States statistics gathered for the year 1999 indicate that foodborne illnesses from bacterial pathogens were responsible for over 76 million cases of reported illnesses, and the loss of work, income, and healthcare visits cost the US economy approximately 7 to 10 billion dollars (Mead *et al.*, 1999). Some of the pathogenic bacteria most commonly reported in cases of foodborne illness include *Campylobacter jejuni*, *Salmonella* spp., *Escherichia coli* O157:H7, *Staphylococcus aureus*, *Clostridium* spp., and *Listeria monocytogenes*. Of these pathogens, *Campylobacter jejuni* and *Salmonella* are the number 1 and 2 most common causative agent of foodborne illnesses commonly associated with poultry meat and poultry consumption in Canada (Public Health Agency of Canada, 2005). In addition, it is important to note that neither of these

bacteria is commonly pathogenic to poultry, and thus chickens merely serve as a reservoir for these important human pathogens. This fact, combined with the foodborne illness statistics, illustrates the need for a comprehensive understanding of the factors which contribute to their persistence, and elimination in the gastrointestinal ecosystem.

2.9.3. Intensive production of poultry

The newly hatched chick's normal colonization resistance is conferred via the establishment of normal gut microflora. However, the normal gut microflora is obtained through the ingestion of healthy adult bird fecal matter, and thus is precluded when hen and chicks are reared separately. Once individual chicks are infected, these pathogenic microorganisms spread rapidly to infect the entire flock. Canadian nation-wide surveys revealed that 76.9% of randomly selected chicken broiler flocks were *Salmonella* spp. positive, *S. enteritidis* being one of the main species (Poppe *et al.*, 1991), and that between 77 and 100% of poultry flocks are *C. jejuni* positive (Munroe *et al.*, 1983). During processing these bacteria may cross-contaminate both chicken meat, and processed chicken products, that are then packaged and sent for retail distribution. A study in the United States revealed that 83% of retail chickens were also *C. jejuni* positive (Kinde *et al.*, 1983).

Through the introduction of rigid hygienic measures, it is possible to breed and rear poultry free of these pathogens, however, the cost to the industry would be prohibitive and economically disastrous. Instead, antibiotic therapy is used extensively for the control of poultry bacterial diseases. This can, however, lead to the development of antibiotic resistance which has become a concern for both human and animal health care practitioners. Furthermore, the Gram-positive normal flora present in the gut is

itself inhibitory to *Salmonella* and *Campylobacter* spp. and is reduced by the use of growth-promoting antibiotics. The reduction in this flora provides pathogens with a competitive edge and may result in overt disease.

2.10. Use of antibiotics in agriculture

Antibiotics have been widely used in the livestock and poultry industries since their discovery more than 50 years ago (Dibner and Richards, 2005), and soon afterwards the beneficial effect of their inclusion in poultry rations was reported by Moore *et al*. (1946) and Stokstad and Jukes (1951). In the years following their discovery and first application, antibiotics used as growth promoters (AGP), have made them an extremely important tool in the efficient production of certain animal species. At therapeutic levels antibiotics help to prevent disease in animals and to treat diseases (Bywater, 2004), however at sub-therapeutic levels in diets, antibiotics improve growth rate and efficiency of feed utilization, reduce mortality and morbidity, and improve reproductive performance (Feed Additive Compendium, 2000).

Although the exact means by which antibiotics improve performance and feed efficiency are not clear, they are thought to reduce the total number and/or total number of species of bacteria in the gastrointestinal tract (Collier *et al*., 2003; Gaskins *et al*., 2002). This hypothesis is supported by the lack of response to antibiotics when they are administered to germ-free animals (Coates *et al.*, 1963), and the reduced benefits from antibiotics when management practices aimed at reducing bacterial exposure are employed. In reducing the microbial load within the intestinal environment, the competition for nutrients and production of toxic metabolites outlined in section 3.5.2 are reduced.

2.10.1. Control of pathogens using medicated feed

As well as improving animal performance, the use of AGP also improves animal health by reducing both clinical and sub-clinical disease caused by poultry pathogens. This indirect benefit of AGP has been demonstrated by the increase in therapeutic antibiotic treatment of enteric diseases in poultry due to the removal of AGP from poultry diets in the EU (Bywater, 2005). In terms of food safety AGP also reduce human pathogens in the gastrointestinal tract of poultry. While the specific reduction in pathogens is not the primary reason for the inclusion of AGPs in poultry diets, they do reduce the intestinal numbers of pathogens as a function of the non-specific reduction in gastrointestinal microbes. This includes the control of pathogenic bacteria such as *Escherichia coli* O157:H7 and *Clostridium perfringens*, (Stanley *et al*., 1996; Verstegen and Williams, 2002). Depending on the antibiotic used however, there have been some reports of increased pathogen shedding when AGPs were administered in feed (Barrow, 1989).

2.10.2. Types of antibiotics and their mode of action

The most common types of antimicrobials used in feed are listed in Table 2.1. Mechanistically, these antibiotics work by inhibiting or interfering with a number of different biochemical pathways including protein synthesis, cell wall synthesis, DNA and RNA synthesis, or folic acid synthesis (Davies and Webb, 2004). The end result is an overall reduction in the number of susceptible microorganisms. The observed effects outlined in Table 2.2 are hypothesized to be the result of 1) inhibition of sub-clinical infections from pathogenic microorganisms, 2) reduction in growth depressing microbial metabolites, 3) reduced competition for nutrients by gastrointestinal

Table 2.1. Antimicrobials commonly used in poultry in Canada (After Boulianne, 1999).

Family	Name of the antibiotic	Use
Aminoglycosides	gentamicin	preventive
	neomycin	curative
	streptomycin	curative
	spectinomycin	curative
Bacitracin	zinc bacitracin	preventive
Betalactamines	amoxicillin	curative
	penicillin	curative
Cephalosporines	ceftiofur	preventive
Macrolides	erythromycin	curative
	lincomycin	curative
Quinolones	enrofloxacin	curative
Streptogramin	virginiamycin	preventive
Sulfamides	trimethroprim-sulfa	curative
	sulfaquinoxaline	curative
Tetracyclines	tetracycline	curative
	chlortetracycline	curative
	oxytetracycline	curative
Ionophores	monensin sodium	preventive
	lasalocid sodium	preventive
	salinomycin sodium	preventive
	naduramycin ammonium	preventive

Table 2.2. Summary of reported physiological, nutritional and metabolic effects of growth promoting antimicrobials (After Gaskins *et al*., 2002).

Physiological	Nutritional	Metabolic
Increase		
Nutrient absorption	Energy retention	Liver protein synthesis
Feed intake	Nitrogen retention	Gut alkaline phosphatase
	Vitamin absorption	
	Trace element absorption	
	Fatty acid absorption	
	Glucose absorption	
	Calcium absorption	
	Plasma nutrients	
Decrease		
Food transit time	Gut energy loss	Ammonia production
Gut wall diameter	Vitamin synthesis	Toxic amine production
Gut wall length		Aromatic phenols
Gut wall weight		Bile degradation products
Fecal moisture		Fatty acid oxidation
Mucosal cell turnover		Fecal fat excretion
		Gut microbial urease

microorganisms, 4) enhanced uptake and utilization of nutrients via the thinner intestinal wall associated with antibiotic-fed animals (Francois, 1962; Gaskins *et al*., 2002; Visek, 1978).

In 1995 93% of all starter diets, 97% of grower diets, and 86% of all finisher diets fed to broiler chickens in the United States contained an AGP and although levels of AGP in broiler rations have declined in the United States since the mid 1990s, presently they are included in approximately 60% of all broiler diets (Chapman and Johnson, 2002). In the United States this decrease may reflect consumer concerns over the perceived health risks to humans, however in European countries legislation has resulted in their discontinued use.

2.11. Future of antibiotics as feed additives

Despite the recognized benefits that AGP provide to both producers and consumers, as far back as 1951 there has been concern over their use because of the issue of antimicrobial resistance (Elam *et al.*, 1951). The use of antimicrobial drugs in food animals, including food producing animals, parallels their use in humans, and many of the concerns about the increase antimicrobial resistance stem from the belief that continual exposure to antibiotics in feed ultimately selects for antimicrobial resistance. This is particularly of concern with antibiotics that are used as animal feed additives and in human medicine (Levy, 2000; Tollefson and Miller, 2000). The observed increase in antimicrobial resistance in food animals is seen as a potential source of antimicrobial resistance genes which may ultimately spread to human pathogenic bacteria, and has sparked much debate and an increase in research examining alternatives to AGP (Bywater, 2004).

2.12. Alternatives methods to control pathogenic microorganisms

The debate over the risks associated with the use of AGP in feed has fueled interest in alternative means to control disease and maintain current production levels. Although research using alternatives to AGP is in its' infancy, some positive results have been obtained. Some of these methods are outlined below.

2.12.1. Probiotics and competitive exclusion cultures

One of the most well characterized means to reduce enteric disease is through the administration of live bacteria, or probiotics. Probiotics have been defined as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance" (Fuller, 1989), and their beneficial effects have been recognized for over a century (Patterson and Burkholder, 2003). In the early 1970s, studies aimed at reducing the ability of *Salmonella* to colonize the intestinal tract of chickens, demonstrated that very effective protection of newly hatched chicks could be achieved with the administration of adult chicken microflora (Nurmi *et al*., 1992; Nurmi and Rantala, 1973; Rantala and Nurmi, 1973). These cultures are known as competitive exclusion cultures, and like probiotics, inhibit the colonization of the chicken intestine by pathogenic bacteria blocking the ability of pathogens to attach to the intestine by occupying binding sites normally occupied by the pathogens (Nurmi *et al.*, 1992). More recently it has been shown that probiotics not only inhibit or block the ability of pathogens to attach to epithelial surfaces, but also limit pathogen colonization through competition for nutrients and production of antibacterial substances such as VFAs and bacteriocins (Corrier *et al.*, 1991; Edens *et al.*, 1997). These are thought to inhibit pathogens by producing environmental conditions which may be suboptimal for their

growth. Recent work has demonstrated that in addition to the above mechanisms, pathogen inhibition may be the result of a more subtle and complex phenomena such as communication between epithelial cells and gastrointestinal microorganisms (Hooper and Gordon, 2001), and modulation of components of the immune system (Jeurissen *et al.*, 2002).

The administration of probiotic organisms to control pathogens in poultry is common practice in some parts of the world (Verstegen and Williams, 2002), especially in Japan and Europe (Patterson and Burkholder, 2003). Much of the research using probiotics has focused on *Lactobacillus* and *Bifidobacterium* spp. This focus is due, in part, to the fact that they are normal inhabitants of the chicken intestine and are thought to play an important role in resistance to infection. These microorganisms have also been shown to be sensitive to stress and respond to it with decreased intestinal numbers (Patterson and Burkholder, 2003). As well as these prokaryotic organisms, it has been shown that eukaryotic organisms, including *Saccharomyces boulardii* and *Saccharomyces cerevisiae*, also have the ability to bind *Salmonella* spp., thus interfering with the attachment of the pathogen to the intestinal wall (Spring *et al.*, 2000). The *in vivo* administration of *Saccharomyces cerevisiae* has also been shown to positively affect performance when administered to broilers (Onifade *et al.*, 1999). In addition to the benefits that *Saccharomyces cerevisiae* administration may have on broiler chickens during the grow out period, the administration of *Saccharomyces boulardii* has been shown to reduce pathogen load during transport to slaughter (Line *et al.*, 1997). It has also been shown that there is a positive effect on the number of probiotic bacteria such

as *Bifidobacterium* and *Lactobacillus* spp. when *Saccharomyces* spp. are administered (Belem and Lee, 1998).

While the administration of one or a few species of probiotic bacteria has been shown to be beneficial, administration of very complex, undefined assemblages of indigenous gut microflora has been shown to be the most effective strategy for probiotic administration. However, the large-scale production of these complex mixtures in a consistent manner has proven to be a difficult challenge. In addition to production difficulties, government approval for complex, undefined, probiotic mixtures has been unable to be secured. This is due, in part, because the underlying protective mechanisms by which probiotics and competitive exclusion cultures work is not fully understood and requires further research. This includes a clear understanding of the microorganisms themselves as well as the gastrointestinal conditions in which they proliferate.

2.12.2. Prebiotics

Prebiotics have also generated a significant amount of interest as a replacement for AGP (Patterson and Burkholder, 2003) and for their ability to improve animal health (Verstegen and Williams, 2002). They have been described as "non digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon leading to an improvement in host health" (Gibson and Roberfroid, 1995). In the context of poultry nutrition, these prebiotics also include soluble and insoluble non-starch polysaccharides (NSP). These NSP represent the main substrates for bacterial fermentation in the hindgut (Choct *et al.*, 1999), and as such they may alter the gastrointestinal microflora by inhibiting the growth of pathogenic species and/or encouraging the growth of beneficial species

(Chung and Day, 2004; Hidaka *et al*., 1990; Mitsuoka *et al*., 1987; Tokunaga *et al*., 1986). Much of the research using prebiotics to alter intestinal microflora has been done in humans and has centered around the use of fructose, mannose, and galactose (Hidaka *et al.*, 1990). In poultry, however, most of the research has been done using mannanoligosaccarides (Daskiran *et al.*, 2004; Sims *et al.*, 2004), fructooligosaccarides (Bailey *et al*., 1991; Oyarzabal and Conner, 1995; Waldroup *et al*., 1993; Xu *et al*., 2003), and isomaltooligosaccarides (Chung and Day, 2004).

Feeding fructooligosaccaharides Bailey *et al*. (1991) demonstrated a reduction in the ability of *Salmonella typhimurium* to proliferate in the gastrointestinal tract of chickens. The observed reduction was enhanced when the authors co-administered a competitive exclusion culture, which is consistent with the research of others (Hinton *et al.*, 1990). The authors demonstrated *in vitro* that *S. typhimurium* did not utilize the fructooligosaccharide as a sole source of carbon, and thus the observed results were due to the fructooligosaccharide being used as substrate by beneficial bacteria which were increased with the addition of the competitive exclusion culture. In addition to fructooligosaccharide, mannanoligosaccharides have been used to reduce cecal colonization by *Salmonella enterica* (Fernandez *et al.*, 2002), however rather than serving as substrate for beneficial intestinal microflora, it has been shown that mannanoligosaccharides are capable of agglutinating certain pathogens *in vitro* . Thus, as well as serving as substrate for growth, some oligosaccharides may also preferentially bind to bacterial surface lectins which may inhibit the ability of pathogens to attach to epithelial cells.

Although the administration of dietary fibre to chickens has been shown to produce beneficial results, research is needed to support the value of prebiotics in the development of a functional product. For example, a more thorough understanding of the mechanisms by which they act to support a healthy gut environment, alone and in combination with other methods used in the control of salmonella and campylobacter in the poultry industry, is needed if they are to see widespread use (Patterson and Burkholder, 2003).

2.12.3. Passive transfer of immunity

2.12.3.1. Hen egg antibodies

A highly attractive and effective alternative approach for the control of potential pathogens present in the intestinal tract is the administration of specific neutralizing antibodies in the diet. The advantage of feeding antibodies with the diet is that they provide a continuous control of potential pathogens and do not result in the development of antibiotic resistant strains of microorganisms. Previous work done in other animal models suggests that hen egg antibodies (HEA) can be effective in the control of some pathogens (Imberechts *et al*., 1997; Marquardt *et al*., 1999; Wiedemann *et al*., 1991; Yokoyama *et al*., 1997; Yokoyama *et al*., 1992). Compared to other antibody sources, vaccination of laying hens provides a cost effective source of antibodies, with egg yolks containing as much as 25 mg of immunoglobulin per mL of yolk (Rose *et al.*, 1974). A laying hen can produce approximately 300 eggs per year and the volume of one egg yolk is approximately 15 mL, which could supply close to 100 g of antibody per hen per year; an amount which has the potential to neutralize a large bacterial population. The egg yolk containing the antibodies can be spray-dried without loss of activity and can be fed directly to the chicken. Administration of HEA in this manner has been shown to be

effective for the treatment of a number enteric pathogens including enterotoxigenic *E. coli* in pigs (Imberechts *et al.*, 1997; Marquardt *et al.*, 1999) and calves (Ikemori *et al.*, 1992), *Salmonella* spp. in calves (Yokoyama *et al.*, 1998a), mice (Peralta *et al.*, 1994; Yokoyama *et al.*, 1998b), and humans (Sugita-Konishi *et al.*, 2000), and *Helicobacter pylori* in humans (Shin *et al.*, 2002). In addition to enteric bacterial infections, HEAs have shown promise in the treatment of viral infections as well (Bartz *et al.*, 1980; Ebina *et al.*, 1990; Hiraga *et al.*, 1990; Kuroki *et al.*, 1993). Although positive results have been obtained for some pathogens in other animal species, very little research however, has been done to assess the ability of hen-egg antibodies to reduce or eliminate enteric pathogens in poultry.

2.12.3.2. Plantibodies

The widespread use of antigen-specific antibodies for prophylaxis and therapy may require the administration of large quantities of antibody, and although present sources of antibody such as hen-egg antibodies may provide a suitable product of relatively high quantity and quality, alternate means for there production have been investigated (Hayden *et al.*, 1997). Recombinant antibody technology may provide the antibody products required by producing them in transgenic plants (Berghman *et al*., 2005; Franconi *et al*., 1999; Stoger *et al*., 2002), or bacteria (Franconi *et al.*, 1999). By far the most economical source of specific antibodies is derived from plants. The the cost of producing 1 gram of purified antibody antibodies in maize estimated to be approximately $0.10 USD (Berghman *et al.*, 2005). Antibodies originally expressed in tobacco plants (Hiatt and Ma, 1992), are now produced in seed crops which in terms of animal feeds is the most practical and economical (Berghman *et al.*, 2005). Importantly,

pathogen-specific recombinant proteins have been expressed as functional secretory IgA (Ma *et al.*, 1995). Realistically, however, consumers are wary of transgenic plants and overcoming this may ultimately be one of the biggest obstacles to research, production, and use in animal feeds.

2.12.4. Bacteriophage

Bacteriophage are viruses that infect and kill bacteria. This bactericidal activity makes them candidates for the control of gastrointestinal bacteria, especially pathogens of both animal and human concern (Huff *et al.*, 2005). Ironically, when bacteriophage were first discovered in the early 1900s their potential as a therapeutic agent was recognized, but the discovery and use of antibiotics resulted in the discontinuation of research on bacteriophage as therapeutics (Barrow and Soothill, 1997). Recent concerns over the use of AGP in feed has now increased interest in using them again to treat intestinal diseases and to reduce the incidence of foodborne illness from infected meat products (Alisky *et al.*, 1998; Huff *et al.*, 2005). They have been shown to be effective in reducing *E. coli* induced diarrhea in calves, piglets, and lambs (Smith and Huggins, 1983, 1982; Smith *et al*., 1987). Using a murine model, they have also been shown to be effective in treating infections due to *E. coli* (Smith and Huggins, 1982), as well as *Enterococcus faecium* (Biswas *et al.*, 2002), *Acinetobacter baumanii* and *Pseudomonas aeruginosa* (Soothill, 1992), and *Staphylococcus aureus* (Matsuzaki *et al.*, 2003). In chickens they have been shown to protect from a lethal injection of *E. coli* (Barrow *et al.*, 1998).

In addition to treatment of bacterial infections, bacteriophage have been shown to be effective in reducing the pathogen load on poultry meat (Goode *et al.*, 2003). Goode *et*

al. (2003) demonstrated that lytic bacteriophage were capable of reducing the number of culturable *Salmonella enteritidis* and *Campylobacter jejuni* on chicken skin when applied to experimentally infected carcasses.

The use of bacteriophage to treat disease may be possible, however, there are some limitations which include their specificity, ability to act as a vehicle for pathogenic genetic material, issues concerning regulatory acceptance, protection of property rights, and practical routes of administration (Huff *et al.*, 2005). Despite these limitations, bacteriophage have the potential to help fight bacterial enteric infections and reduce the incidence of foodborne illness.

2.12.5. Natural antimicrobials

Many natural products have observed antimicrobial activity and have thus been used to preserve foods for centuries, and their ability to inhibit the growth of intestinal pathogens has been examined. These include berries and their phenolics which have been shown to be inhibitory to some intestinal pathogens (Puupponen-Pimia *et al*., 2005; Rauha *et al*., 2000), tannic acid (Kubena *et al.*, 2001), essential oils from various plants including aniseed, rosemary, and celery (Elgayyar *et al*., 2001; Mitsch *et al*., 2004; Verstegen and Williams, 2002). Although in principle these compounds have been shown to be inhibitory towards intestinal pathogens, research has yet to be done which demonstrates their commercial suitability.

2.12.6. Organic acids

Organic acids or volatile fatty acids (VFA) were originally added to animal feed to inhibit fungal growth (Paster, 1979), however competitive exclusion studies in poultry implicated these same compounds in the cecae of chickens with reduced *Salmonella*

counts (Barnes *et al.*, 1979; Corrier *et al.*, 1994; Corrier *et al.*, 1995). Subsequent *in vitro* research examined the ability of VFAs to inhibit the intestinal microflora of poultry and confirmed that they do have antibacterial activity. Although the exact mechanisms by which VFAs inhibit intestinal microflora are unclear (Ricke, 2003), undissociated VFAs are thought to penetrate the cell membrane of bacteria and dissociate in the more alkaline cytoplasm, hereby increasing the inward leak of protons so that efflux of protons is not rapid enough to alkalinize the cytoplasm (Cherrington *et al*., 1991; Kashket, 1985). Species that have been shown to be sensitive to various organic acids include pathogens of both human and animal concern such as *Salmonella* and *E. coli* (Hinton and Linton, 1988; Izat *et al*., 1990), as well as *Campylobacter* (Chaveerach *et al.*, 2004). *In vivo* studies examining the effect of VFAs on the gastrointestinal microflora intestinal pathogens have most often used formic acid (Hinton and Linton, 1988; McHan and Shotts, 1992) and propionic acid (Hinton and Linton, 1988; Hume *et al*., 1993; McHan and Shotts, 1992), however other VFAs have included acetic acid and hydrochloric acid (Chaveerach *et al.*, 2002). In addition to reduced growth in the presence of VFAs, van Immerseel *et al.* (2003) showed that the ability of *Salmonella enteritidis* to invade chicken epithelial cells *in vitro* is influenced by the VFAs.

The promising results obtained from research has resulted in the development of a number of commercial products, however more research is required to improve their effectiveness. Of particular interest is the phenomenon of acid tolerance, including the stress response and virulence factors which allow for resistance in more acidic environments (Ricke, 2003).

2.12.7. Diet

Using percent G+C to characterize diet-dependant differences in the gastrointestinal microflora, Apajalahti *et al*. (2001) examined a number of Finnish poultry farms. They found that when the gastrointestinal profiles from different farms were compared, diet turned out to be the strongest determinant in of the cecal bacterial community structure. To assess the affect that diet has on the microbial ecology of the gastrointestinal tract, a number of different dietary factors have been examined for their ability to influence the gastrointestinal microflora.

Processing of feed has been shown to affect the microbial ecology and pathogen load of the intestine (Apajalahti *et al*., 2001; Engberg *et al*., 2002). Engberg *et al*. (2002) examined the influence of pelleting *versus* mash feeds and demonstrated that simple factors such as particle size can alter the intestinal populations of several microbial species, most notably the number of *Clostridium* spp., with pellet-fed birds having a lower number of intestinal *Clostridium* spp.. In addition to feed processing, the influence of dietary constituents have on the intestinal microflora have also been examined.

Dietary protein has been shown to influence the intestinal numbers of a number of pathogenic bacteria including *C. perfringens* (Drew *et al.*, 2004) and *C. jejuni* (Udayamputhoor *et al.*, 2003). This may be a function of the amino acid profile of various proteins which may upregulate specific virulence factors in pathogenic bacteria such as *S. enteritidis* (Durant *et al.*, 2000) or are immunomodulatory and influence the ability of a pathogen to cause disease (Takahashi *et al.*, 1997), however high protein

diets must certainly also alter the intestinal microflora by preferentially increasing proteolytic members.

In addition to the inclusion of carbohydrate prebiotics outlined above, the type of dietary grain has been shown to influence the intestinal microflora, including pathogens (Annett *et al*., 2002; Guo *et al*., 2004; Guo *et al*., 2003; Phillips *et al*., 2004; Riddell and Kong, 1992). As with dietary protein, this may be due to the positive selective pressure that the type and level of dietary carbohydrates found in grain may exert on the saccharolytic members of the chicken intestinal microflora. However, there also appears to be an immunomodulatory effect from certain polysaccharides even at low inclusion rates (Benson *et al*., 1993; Chen *et al*., 2003; Gulsen *et al*., 2002; Kleessen *et al*., 2003; Takahashi *et al*., 2000).

The dietary fat added to poultry diets has been shown to not only have an effect on bird performance (Golian and Maurice, 1992), but also intestinal populations in broiler chickens (Danicke *et al.*, 1999). Considerable research has shown that there is a link between dietary lipids and the immune status of chickens (Konjufca *et al*., 2004; Mandal *et al*., 2004; Puthpongsiriporn and Scheideler, 2005; Sijben *et al*., 2003), however as a consequence of fat digestion, fatty acids and monoglycerides are liberated which have been shown to have antibacterial effects in rats (Sprong *et al.*, 1999). Sprong *et al*. (1999) observed that rats fed diets high in bovine milk had reduced levels of *L. monocytogenes* in experimentally challenged animals, but not *S. enteritidis*. In poultry, the effect of dietary fat has been shown to reduce intestinal colonization by pathogens such as *C. perfringens* (Knarreborg *et al.*, 2002). Despite these positive findings however, little research has been done which examines the relationship

between type, lipid profile, and the level of fat inclusion and the microbial ecology of the chicken gastrointestinal tract.

As well as the above dietary components, the addition of exogenous enzymes to broiler diets has been shown to affect the gastrointestinal microbial populations. Dietary enzymes shown to influence the microbial ecology of the chicken intestine include β-glucanase (Choct *et al.*, 1999) and xylanase (Hubener *et al.*, 2002). Although the effect of exogenous enzymes on pathogenic bacteria has not been extensively examined, the addition of xylanase has been shown to reduce intestinal colonization by clostridia, salmonella, and *C. jejuni* (Apajalahti and Bedford, 1998; Fernandez *et al*., 2000).

Clearly, the observation that dietary components and processing can significantly alter the gastrointestinal environment and microflora underscores the potential that these factors may have in controlling gastrointestinal pathogens for improved bird health and performance, and thus should be examined further.

2.13. Conclusions

Intensification of poultry production systems combined with consumer fears over antibiotic residues and resistance have raised the need to find alternatives to AGP in feed. To date, the use of probiotics and various feed ingredients or additives which favor the growth of beneficial microorganisms at the expense of human and animal pathogens have proven less than satisfactory under all conditions, and there is a gap in our current understanding of the factors which contribute to the functioning of gastrointestional ecosystem. In addition to the need for more sophisticated methods for the detection and enumeration of the community members within the gastrointestinal mileu, there is an equal deficiency in our current understanding of the roles that the

various community members have both within the community and in the gastrointestinal ecosystem as a whole. In terms of intervention strategies, it is beginning to become clear is that there does not appear to be a single treatment that will be able to replace AGPs. Ultimately any effective management strategy may require the use of a number of treatments which are either being presently researched or currently in use, but with mixed results.

Based on these observations, the overall objective of these studies was to investigate the effect of probiotics, hen egg antibodies and dietary proteins on intestinal colonization by three model pathogens; *Salmonella enteritidis*, *Campylobacter jejuni* and *Clostridium perfringens*.

3. THE EFFECT OF ADMINISTRATION OF *LACTOBACILLUS FERMENTUM* AND *BIFIDOBACTERIUM ANIMALIS* FOR THE CONTROL OF *SALMONELLA ENTERITIDIS* AND *CAMPYLOBACTER JEJUNI* IN BROILER CHICKENS

3.1. Abstract

Salmonella enteritidis and *Campylobacter jejuni* are the most prevalent human pathogens associated with poultry meat consumption. In this study, 10 *Lactobacillus* and 6 *Bifidobacterium* isolates were screened for their survivability in increasing concentrations of bile and decreasing pH. Based on these results, two model probiotics (*Lactobacillus fermentum* and *Bifidobacterium animalis*) were assessed in *in vivo* trials for their ability to inhibit the intestinal colonization of day-of-hatch broiler chicks by *Salmonella enteritidis* and *Campylobacter jejuni*. Compared to the negative control groups, administration of either *L. fermentum* or *B. animalis* via oral gavage for 7 consecutive days did not significantly reduce ($P < 0.05$) the ileal or cecal colonization by either pathogen in experimentally challenged chicks. Although not statistically significant, administration of *L. fermentum* did result in fewer *S. enteritidis* positive livers and spleens in experimentally challenged chicks. These results suggest that although the intestinal survival of probiotic bacteria is essential for their use in feed supplements, it should not be used as the sole criterion for probiotic strain selection. In

addition, despite the fact that *L. fermentum* did not reduce ileal or cecal colonization by *S. enteritidis* using our infection model, there is some evidence that *L. fermentum* might reduce translocation of pathogens to spleen and liver.

3.2. Introduction

Modern poultry production practices leave chicks susceptible to colonization by a wide variety of both human and avian pathogens. The newly hatched chick's normal colonization resistance is conferred via the establishment of normal gut microflora, however, the normal gut microflora is obtained by ingestion of healthy adult bird fecal matter. The separation of hen and chicks, as is the practice in the modern poultry industry, results in a delay in the development of the normal healthy adult microflora, during which time pathogenic microorganisms may become established in susceptible birds (Bailey *et al.*, 1994). Once birds are infected, the pathogens may be horizontally transferred within the flock and barns, which makes their complete eradication a difficult task. Furthermore, human pathogens such as *Salmonella enteritidis* (Bailey *et al.*, 1994) and *Campylobacter jejuni* (Craven *et al.*, 2001) may become established in the intestinal tract of chickens. Typically these bacteria do not cause disease in poultry and thus their presence may go undetected in birds (Garriga *et al.*, 1998). For example, Canadian nation-wide surveys have revealed that 76.9% of randomly selected chicken broiler flocks were *Salmonella* spp. positive, *S. enteritidis* being one of the main species (Poppe *et al.*, 1991), and that between 77 and 100% of poultry carry *C. jejuni* (Munroe *et al.*, 1983). Cross-contamination during processing may result in these pathogens being spread from intestinal contents to chicken meat, which is then packaged and sent

for retail distribution. A study in the United States revealed that 83% of retail chickens were *C. Jejuni* positive (Kinde *et al.*, 1983).

According to the Public Health Agency of Canada, (2003), *Campylobacter jejuni* and *Salmonella* spp. are the number 1 and 2 most common foodborne pathogens associated with poultry consumption in Canada. In the year 2000, the number of reported cases of Campylobacterosis and Salmonellosis in Canada was 12,352 and 5655, respectively (Public Health Agency of Canada, 2003). These statistics represent a significant cost to the Canadian economy and illustrate the need for a more thorough understanding of the factors which contribute to the persistence of pathogenic microorganisms in poultry, as well as means for their elimination from the gastrointestinal tract.

Probiotics have been defined as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance" (Fuller, 1989), and their administration to control pathogens in poultry is common practice in some parts of the world (Verstegen and Williams, 2002), especially in Japan and Europe (Patterson and Burkholder, 2003). In North America, however, a lack of solid scientific research clearly demonstrating a beneficial effect, as well as an inability to obtain government approval, has somewhat limited their widespread use (Verstegen and Williams, 2002; Patterson and Burkholder, 2003). Much of the research using probiotics has focused on *Lactobacillus* and *Bifidobacterium* spp. due in part to the fact that they are normal inhabitants of the chicken intestine and are thought to play an important role in resistance to infection, but also because these populations appear to be sensitive to stress, responding to it with decreased intestinal numbers (Patterson and

Burkholder, 2003). *Lactobacillus*, *Bifidobacterium* and other lactic acid bacteria (LABs) are thought to aid in the prevention of disease, and exclusion of pathogenic microorganisms by competing for attachment sites on epithelial cells (competitive exclusion) (Fuller, 1977), production of antimicrobial compounds such as organic acids, hydrogen peroxide, and bacteriocins (Servin, 2004), and stimulation of the immune system (Perdigon *et al.*, 2001). Although convincing results have been obtained *in vitro*, similar results *in vivo* have been more difficult to demonstrate. Thus, the objective of these studies was to evaluate the ability of *Lactobacillus fermentum* and *Bifidobacterium animalis* to reduce the intestinal colonization by *Campylobacter jejuni* and *Salmonella enteritidis* in day-of-hatch broiler chickens.

3.3. Methods and Materials

3.3.1. Pathogen selection and culture conditions

A *Salmonella enteritidis* isolate of chicken origin was obtained from Dr. Manuel Chirino, Dept. of Veterinary Microbiology, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK., Canada. As a selective marker for use in challenge studies, the *S. enteritidis* isolate was screened for both Nalidixic Acid and Novobiocin resistance (*S. enteritidis*^{Nal,Novo}). Briefly, the *S. enteritidis*^{Nal,Novo} mutant was obtained by growing the original *S. enteritidis* isolate for 6 hours aerobically at 37ºC in Trypticase Soy Broth (TSB) (Difco, VWR International, Mississauga ON, Canada). The cells were harvested by centrifugation (3640 x g for 15 min at 4ºC), washed twice in phosphate buffered saline (PBS, pH 7.2), and resuspended in a minimal amount of PBS. The cells were then plated on Trypticase Soy Agar (TSA) containing 25µg/mL Nalidixic Acid (Sigma, Sigma-Aldrich, Oakville ON, Canada). The Nalidixic

Acid resistant colonies capable of growth were subsequently transferred to TSA containing 25µg/mL Novobiocin (Sigma, Sigma-Aldrich, Oakville ON, Canada). This resulted in 20 double antibiotic resistant isolates. The growth curves of the 20 double mutants were compared to the original isolate in an *in vitro* experiment. Briefly, the original *S. enteritidis* isolate and all 20 *S. enteritidis*^{Nal,Novo} mutants were grown for 6 hours at 37ºC in TSA and TSA containing 25µg/mL Nalidixic Acid and Nalidixic Acid, respectively. The titre of these cultures were adjusted to ~ 1.5 x 10^2/mL, with 1.0 mL used to inoculate 50 mLs of the appropriate media. Based on the growth curves, a single isolate was chosen and used for all subsequent *in vivo* trials. A Nalidixic Acid resistant (125µg/ml) *C. jejuni* (*C. jejuni*^{Nal}) isolate of human origin was obtained from Dr. Dianne Taylor, Dept. of Microbiology, University of Alberta, Edmonton, AB, Canada, and was used for all *in vivo* trials using *Campylobacter jejuni*. The *S. enteritidis*^{Nal,Novo} mutant was grown aerobically in Trypticase Soy Broth (Difco, VWR International, Mississauga ON, Canada) at 37ºC. *Lactobacillus fermentum* cultures were anaerobically grown in MRS broth (Difco, VWR International, Mississauga ON, Canada) at 37°C. *Bifidobacterium animalis* cultures were anaerobically grown in TPY broth (pH 6.5) at 37°C. The TPY broth consisted of (per litre) 10.0g trypticase, 5.0g phytone, 5.0g glucose, 2.5g yeast extract, 0.5g cystein-HCl, 2.0g K_2HPO_4, 0.5g $MgCl_2$, 0.25g $ZnSo_4$, 0.15g $CaCl_2$, 0.03 $FeCl_3$, 1.0mL Tween-80. For all bacteria except *C. jejuni*, cultures were grown until late log phase (6 hours for *S. enteritidis,* overnight for *L. fermentum* and *B. animalis*), harvested by centrifugation (3640 x g for 15 min at 4ºC), washed twice and resuspended in phosphate buffered saline (PBS, pH 7.2) to a final titre of 1 X 10^8 CFU/mL. The *C. jejuni* (Nal^r) isolate was grown on blood agar plates containing

125µg/ml Nalidixic acid, aseptically scraped from plates, washed twice and resuspended in PBS as above.

3.3.2. Probiotic selection and culture conditions

To select a candidate probiotic strain of *Lactobacillus*, 7 isolates were obtained from the cecum of an adult laying hen reared in a battery cage. Using API CHL Medium and API 50 CH strips (bioMérieux Canada, St. Laurent QC, Canada) as per the manufacturers instructions, the presumptive *Lactobacillus* spp. isolates were positively identified as *L. fermentum* (two isolates), *L. acidophilus* (two isolates), *L. delbrückii* (two isolates), and *L. salivarius*. In addition to the cecal isolates, 3 commercial cultures were used; a lyophilized commercial strain of *L. acidophilus* (Inst. Rosell-Lallemand, Montréal QC, Canada) and *L. reuteri* (BioGaia Biologics #11284) (BioGaia Biologics, Stolkholm, Sweden), as well as an ATCC strain (ATCC, Manassas VA, USA) of *L. amylovorus* (33198). The *Bifidobacterium* spp. candidates were chosen from ATCC stains; *B. animalis* (27536), *B. suis* (27533), *B. thermophilum* (25525), and *B. pseudolongum* (25526) or commercial strains; *B. longum* (Chr. Hansen #701967) and *B. bifidum* (Inst. Rosell #75118). To select isolates most capable of withstanding the harsh conditions in the proximal intestine, all isolates were screened for acid and bile tolerance by exposure to increasing concentrations of porcine bile extract, and decreasing pH. *Lactobacillus* spp. and *Bifidobacterium* spp.. Bacterial cells were harvested as above, with 1.0 mL of each resuspended culture added to 9.0 mLs of either MRS or TPY broth for *Lactobacillus* spp. and *Bifidobacterium* spp., respectively. To assay the bile resistance of the isolates the MRS and TPY broth contained 0.0, 0.05, 0.10, 0.20, 0.40, 0.60, 0.80, or 1.0% porcine bile extract (Sigma, Sigma-Aldrich,

Oakville ON, Canada). To assay the survivability of the isolates in low pH, the resuspended cells were also used to inoculate MRS and TPY broth which had the pH adjusted to 6.0, 5.5, 5.0, 4.5, 4.0, 3.5, 3.0, and 2.5 using HCl. The positive or negative growth was determined by comparing the number of cells in each tube at hour 0 compared to growth after 24 hours growth, based on duplicate plating of cultures on the appropriate media.

3.3.3. Birds and infection

Experimental protocols were approved by the Animal Care Committee of the University of Saskatchewan and were performed in accordance with the recommendations of the Canadian Council on Animal Care (1993). Experimental birds were housed in heated battery cages at the Animal Care Facility of the Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK. In all experiments day-of-hatch Ross 308 mixed-sex broiler chicks (Lilydale Hatchery, Wynyard SK) were used.

3.3.4. Animal challenge experiments

To assess the ability and colonization dynamics of the *S. enteritidis*^{Nal,Nov} and *C. jejuni*^{Nal} isolates in the gastrointestinal tract of day-of-hatch chicks, two separate experiments were conducted. In the first trial, 37 chicks were orally gavaged with 0.09 mLs of an overnight culture of *S. enteritidis*^{Nal,Nov}, placed in the same cage and provided a commercial, non-medicated starter crumble (Co-op Feeds, Saskatoon, SK) and water *ad libitum*. At hours 0, 1, 2, 3, 6, 9, 12, 15, 18, 21, 24, two birds were euthanized by cervical dislocation and the contents from the jejunum, ileum and cecae enumerated as described below. In addition to the first 24 hours, at days 4, 7, and 14, an additional 5

birds were euthanized with the contents enumerated for *S. enteritidis*. For the first 24 hours the intestinal contents were removed by rinsing the intestinal segments with 1.0 mL of sterile PBS., whereas at days 4, 7, and 14 contents from each intestinal length was collected as described below. A second trial was conducted in which all experimental procedures were as described above except that the birds were orally gavaged with *C. jejuni*^{Nal} .

To determine the ability of *L. fermentum* and *B. animalis* to reduce intestinal colonization by either *S. enteritidis* or *C. jejuni*, 4 separate trials were conducted in duplicate. In each trial 40 day-of-hatch broiler chicks were randomly assigned to either the experimental or control group. Both groups were orally gavaged with 0.9 mLs of a ~1.0 X 10^8 CFU/mL resuspended culture of either *S. enteritidis* or *C. jejuni* as prepared above. The challenged chicks were provided a non-medicated starter crumble (Co-op Feeds, Saskatoon, SK) and water *ad libitum*. Three hours post-challenge, the chicks in the treatment group were administered the probiotic by orally gavaging 0.9 mLs of a ~1.0 X 10^8 CFU/mL resuspended culture of either *L. fermentum* or *B. animalis* as prepared above. In each trial the chicks in the control group were orally gavaged with an equal volume of sterile PBS. The administration of the probiotic or PBS continued until day 7 of the experiment. On day 14 the birds were euthanized with ileal and cecal contents collected and plated as below. In trials where birds were challenged with *S. enteritidis*, the liver and spleen were aseptically removed and plated as below.

3.3.5. Sample collection and pathogen enumeration

In all experiments, the entire contents of the ileum and cecae were separately removed from each bird. The collected contents from each gut location was then mixed

thoroughly and a subsample of each was placed in sterile peptone containing 0.5% cysteine hydrochloride and immediately placed on ice. The subsamples were serially diluted and plated in duplicate using an automated spiral plater (Autoplate, Spiral Biotech Inc., Bethesda MD, USA). For the enumeration of *C. jejuni*, samples were plated on Modified Preston's Campylobacter Isolation Agar (Oxoid) containing 125µg/ml Nalidixic acid (Sigma) and incubated at 37°C for 48 hours in the appropriate atmospheric conditions (BBL271045 Campy-Pac; Becton Dickinson Microbiology Systems). Enumeration of *S. enteritidis* was done using Brilliant Green Agar (Difco) containing 25 µg/ml of Nalidixic acid and Novobiocin (Sigma), incubated for 16 hours aerobically. For experiments where birds were challenged with *S. enteritidis*, the spleen and liver for each bird was also aseptically collected, placed in sterile peptone containing 0.5% cysteine hydrochloride and immediately placed on ice. The spleen and liver was then diluted 1:10, homogenized (Brinkmann PT 10-35), and plated on Brilliant Green Agar (Difco) containing 25 µg/ml of Nalidixic acid and Novobiocin.

3.3.6. Statistical analysis

Individual birds were considered the experimental unit in all experiments. Bacterial numbers at each anatomical site were log transformed to normalize them prior to analysis. Statistical analyses of $\log_{10}$ transformed colony forming units were analyzed using the general linear model of SPSS (v.10.0.5, SPSS Inc, Chicago IL, USA). Treatment means were compared using the Ryan-Einot-Gabriel-Welsch multiple F test, and were considered significant when $P < 0.05$.

3.4. Results

Screening the *S. enteritidis* isolate for double antibiotic resistance resulted in 20

isolates capable of growing in 25µg/mL Novobiocin and Nalidixic acid. Although all 20 isolates grew well compared to the original isolate, numbers 19 and 20 appeared to be equally fast growing compared to the original isolate (Table 3.1). Of these 2, #19 was chosen for all *in vivo* challenge studies. The ability of both pathogenic strains of bacteria to colonize and become established in the gastrointestinal tract of day old chicks was determined. The antibiotic resistant markers permitted us to monitor and follow population dynamics within the intestinal tract of challenged birds without any apparent loss in virulence. When birds were challenged with both *S. enteritidis* and *C. jejuni*, the pathogens were present in the entire gastrointestinal tract from 3 hours onward (Figures 3.1 and 3.2, respectively). In addition, intestinal colonization by *S. enteritidis* and *C. jejuni* was measured at days 4, 7, and 14 after the oral challenge (Figures 3.3 and 3.4, respectively). Thus the 3 hour time period required by both pathogens to become present in all compartments of the gastrointestinal tract of chicks determined the time at which all probiotics were initially administered in all *in vivo* trials.

Based on their ability to grow in bile and low pH (Tables 3.2 and 3.3), *L. fermentum* and *B. animalis* were chosen as model probiotics. In addition to the ability of these two isolates to grow either as well as, or better, than the other strains. both were also of chicken origin. In replicated trials, both *L. fermentum* and *B. animalis* were unable to reduce or eliminate the level of either ileal or cecal colonization by *C. jejuni* (Tables 3.4 and 3.5, respectively) or *S. enteritidis* (Tables 3.6 and 3.7, respectively). Administration of *B. animalis* or *L. fermentum* did not significantly reduce the translocation of *S. enteritidis* to the spleen or liver.

Table 3.1. Table showing the growth of double-antibiotic resistant *S. enteritidis* mutants compared to the non-antibiotic resistant original *S. enteritidis* strain.

	Time (hours)					
Isolate	0	2	4	6	8	12
original	2.16[1]	4.70	7.81	8.65	8.64	8.20
1	2.20	3.41	7.15	8.78	8.32	7.78
2	2.17	4.47	7.28	8.79	7.99	7.89
3	2.18	3.28	7.00	8.82	8.87	7.33
4	2.14	4.47	6.90	8.84	8.82	7.95
5	2.20	4.01	7.01	8.80	8.32	7.95
6	2.26	4.00	7.00	8.78	8.54	7.99
7	2.22	3.23	5.30	8.82	8.81	8.56
8	2.19	4.30	7.30	8.81	8.32	8.65
9	2.16	3.75	5.48	8.81	7.89	8.34
10	2.14	3.95	6.95	8.80	8.72	7.35
11	2.18	3.00	5.00	8.78	8.70	8.00
12	2.21	4.88	6.88	8.79	8.54	8.33
13	2.20	4.36	7.00	8.79	8.78	7.91
14	2.17	3.75	6.78	8.83	8.65	7.99
15	2.19	4.31	7.00	8.79	8.78	8.35
16	2.22	3.48	7.00	8.79	8.66	8.07
17	2.18	3.40	6.95	8.84	8.01	7.95
18	2.17	4.04	6.65	8.78	8.78	8.00
19[2]	2.20	4.95	7.95	8.84	8.74	8.04
20	2.16	4.98	7.90	8.78	8.60	8.28

[1] Values are the $\log_{10}$ transformed CFU /mL average of two replicate cultures.
[2] denotes isolate chosen for all *in vivo* and *in vitro* experiments .

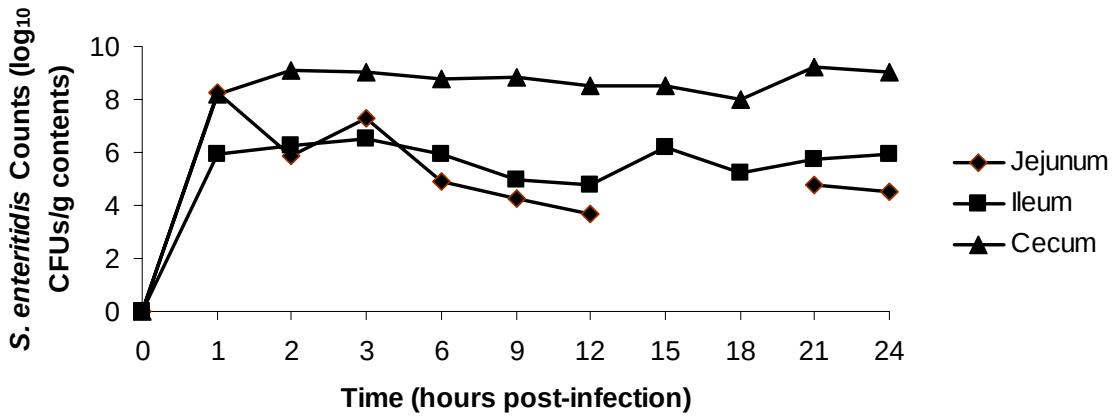

Figure 3.1. Graph showing the temporal distribution of the Nal^r/Novo^r strain of *Salmonella enteritidis* in day-of-hatch Ross 308 broiler chicks.

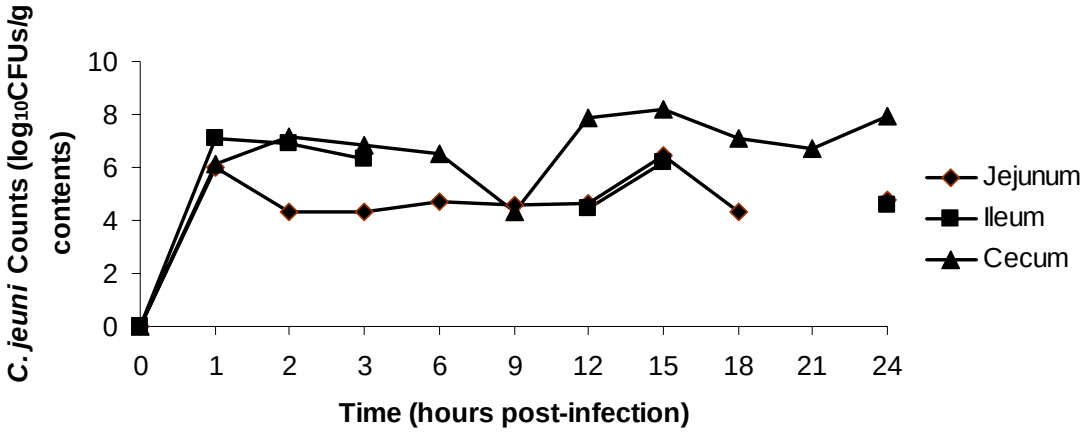

Figure 3.2. Graph showing the temporal distribution of the Novo^{r} strain of *Campylobacter jejuni* in day-of-hatch Ross 308 broiler chicks.

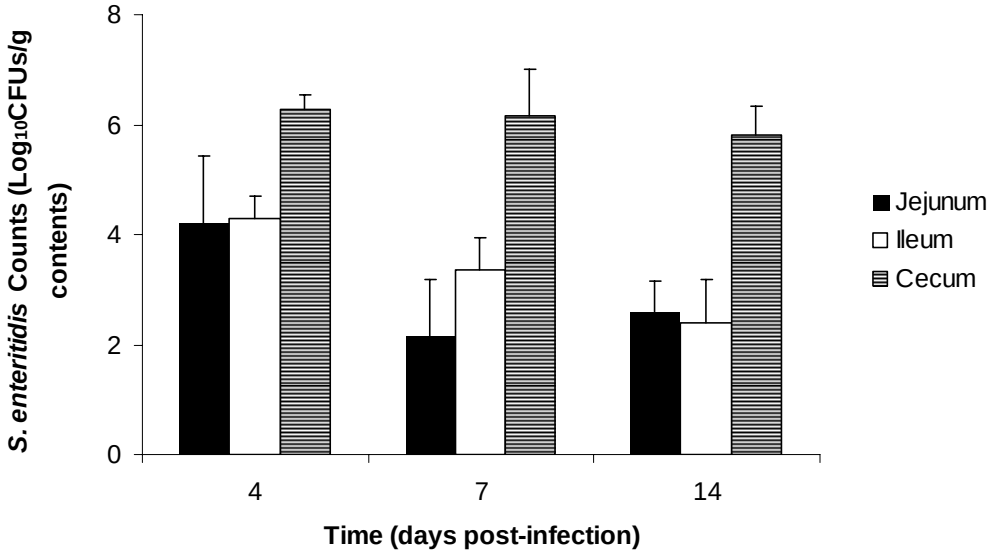

Figure 3.3. Graph showing jejunal, ileal, and cecal colonization by the Nal^r/Novo^r strain of *Salmonella enteritidis* broiler chickens. Bars represent the mean ± SEM of 5 birds euthanized at days 4, 7 and 14.

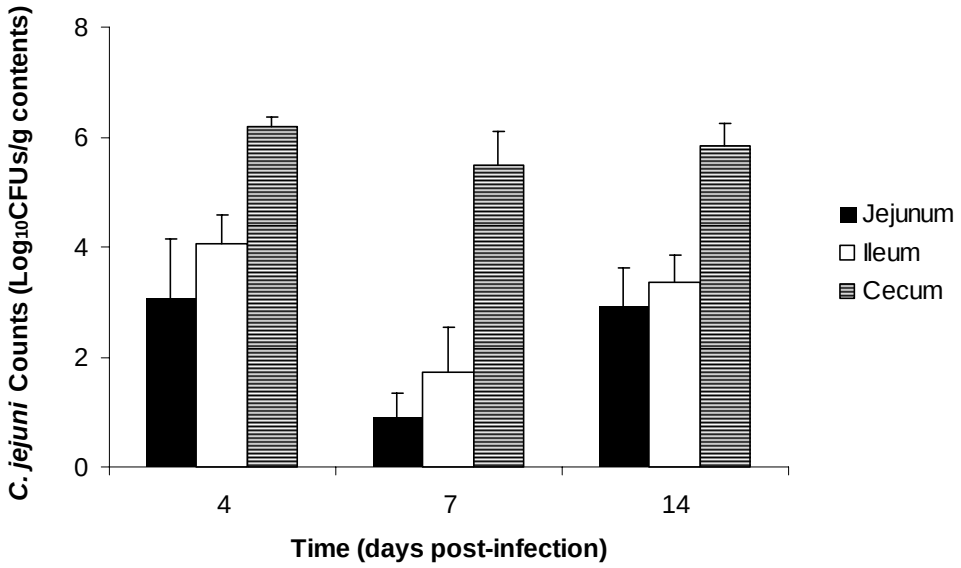

Figure 3.4. Graph showing jejunal, ileal, and cecal colonization by the Novo^{r} strain of *Campylobacter jejuni* in broiler chickens. Bars represent the mean ± SEM of 5 birds euthanized at days 4, 7 and 14.

Table 3.2. The effect of bile concentration and pH on the ability of *Lactobacillus* spp. to grow in MRS broth.

Lactobacillus Strain	Bile Concentration (%) / pH							
	0.0/6.0	.05/5.5	0.1/5.0	0.2/4.5	0.4/4.0	0.6/3.5	0.8/3.0	1.0/2.5
L. fermentum (A)[2]	+ / +[1]	+ / +	+ / +	+ / +	+ / +	- / +	- / +	- / -
L. fermentum (B)	+ / +	+ / +	+ / +	+ / -	- / -	- / -	- / -	- / -
L. acidophilus (A)	+ / +	+ / +	+ / +	- / +	- / +	- / +	- / -	- / -
L. acidophilus (B)	+ / +	+ / +	+ / +	+ / +	- / +	- / +	- / -	- / -
L. delbrückii (A)	+ / +	+ / +	+ / +	- / -	- / -	- / -	- / -	- / -
L. delbrückii (B)	+ / +	+ / +	+ / +	+ / +	- / -	- / -	- / -	- / -
L. salivarius	+ / +	+ / +	+ / +	+ / +	- / +	- / -	- / -	- / -
L. acidophilus (Inst. Rosell)	+ / +	+ / +	+ / +	+ / +	- / +	- / -	- / -	- / -
L. reuteri (BioGaia Biologics))#11284)	+ / +	+ / +	+ / +	+ / -	- / -	- / -	- / -	- / -
L. amylovorus (ATCC #33198)	+ / +	+ / +	+ / +	+ / -	- / -	- / -	- / -	- / -

[1]Cultures were incubated anaerobically overnight at 37ºC, with positive or negative growth represented by a + or – sign, respectively.
[2]Designates the strain chosen for all *in vivo* and *in vitro* experiments.

Table 3.3. The effect of bile concentration and pH on the ability of selected *Bifidobacterium* spp. to grow in TPY broth.

	Bile Concentration (%) / pH							
Bifidobacterium Strain (ATCC #)	0.0/6.0	.05/5.5	0.1/5.0	0.2/4.5	0.4/4.0	0.6/3.5	0.8/3.0	1.0/2.5
B. animalis (27536)[2]	+ / +[1]	+ / +	+ / +	+ / +	+ / +	+ / +	- / -	- / -
B. suis (27533)	+ / +	+ / +	+ / +	+ / +	- / -	- / -	- / -	- / -
B. thermophilum (25525)	+ / +	+ / +	- / -	- / -	- / -	- / -	- / -	- / -
B. pseudolongum (25526)	+ / +	+ / +	+ / +	+ / +	+ / -	- / -	- / -	- / -
B. longum (701967)	+ / +	+ / +	+ / +	+ / +	+ / +	+/ +	- / -	- / -
B. bifidum (75118)	+ / +	+ / +	+ / +	+ / +	+ / -	- / -	- / -	- / -

[1]Cultures were incubated anaerobically overnight at 37ºC, with positive or negative growth represented by a + or – sign, respectively.
[2]Designates the strain chosen for all *in vivo* and *in vitro* experiments.

Table 3.4. Trial 1 and 2 mean Log_{10} CFU counts of *Campylobacter jejuni* in ileum and cecum of 14 day old broiler chickens with and without administration of *Lactobacillus fermentum*.

	Ileum	Cecum
Trial 1		
No probiotic	3.63	4.39
L. fermentum	3.62	5.00
Pooled SEM	0.43	0.47
Trial 2		
No probiotic	3.92	4.79
L. fermentum	3.79	4.64
Pooled SEM	0.44	0.47

`

Table 3.5. Trial 1 and 2 mean Log_{10} CFU counts of *Campylobacter jejuni* in ileum and cecum of 14 day old broiler chickens with and without administration of *Bifidobacterium animalis*.

	Ileum	Cecum
Trial 1		
No probiotic	3.55	3.71
B. animalis	2.72	3.84
Pooled SEM	0.44	0.47
Trial 2		
No probiotic	3.28	3.71
B. animalis	3.18	3.99
Pooled SEM	0.47	0.46

Table 3.6. Mean Log_{10} CFU counts of *Salmonella enteritidis* in ileum, cecum, liver and spleen of 14 day old broiler chickens with and without administration of *Lactobacillus fermentum*.

	Ileum	Cecum	Liver	Spleen
Trial 1				
No probiotic	3.89	4.86	1.28 (9/19)[1]	1.76 (12/19)
L. fermentum	4.07	5.01	1.23 (6/18)	1.46 (8/18)
Pooled SEM	0.34	0.43	0.22	0.23
Trial 2				
No probiotic	3.96	4.90	0.93 (9/20)	1.27 (10/20)
L. fermentum	3.96	4.90	0.90 (6/19)	1.04 (7/19)
Pooled SEM	0.41	0.43	0.20	0.23

[1]Values in brackets represent the number of *S. enteritidis* positive birds/total number of birds sampled

Table 3.7. Mean Log_{10} CFU counts of *Salmonella enteritidis* in ileum, cecum, liver and spleen of 14 day old broiler chickens with and without administration of *Bifidobacterium animalis*.

	Ileum	Cecum	Liver	Spleen
Trial 1				
No probiotic	3.61	4.46	0.90 (7/19)[1]	1.35 (9/19)
B. animalis	3.47	4.89	0.75 (8/19)	1.44 (10/19)
Pooled SEM	0.40	0.43	0.20	0.24
Trial 2				
No probiotic	3.82	4.44	0.93 (9/20)	1.27 (10/20)
B. animalis	3.67	5.00	1.00 (6/19)	1.51 (7/19)
Pooled SEM	0.41	0.44	0.20	0.23

[1]Values in brackets represent the number of *S. enteritidis* positive birds/total number of birds sampled

3.5. Discussion

The ability to overcome the physico-chemical hurdles such as bile acid and low pH in the gastrointestinal tract dictates to a large extent the survival and subsequent proliferation of probiotic bacteria in the gastrointestinal tract (Fooks and Gibson, 2002b). These selective pressures define which microorganisms will ultimately colonize the gut, and are most suited to competition with other microorganisms, especially pathogens (Garriga *et al.*, 1998). In addition to being able to survive these hurdles, host specificity has been recognized and well documented (Barrow *et al*., 1980; Fuller, 1975; Suegara *et al*., 1975) Thus, in these studies isolation and selection of *Lactobacillus* and *Bifidobacterium* species was undertaken as the first step in assessing the suitability of these microorganisms as a probiotic for the reduction of intestinal colonization by either *C. jejuni* or *S. enteritidis*.

Based on our results there were clear inter and intra-species differences in the ability of different strains of *Lactobacillus* and *Bifidobacterium* to grow in the presence of differing concentrations of bile, and at different pH. In some cases, however, strains grew equally well compared to *B. animalis* and *B. bifidum*. Thus, in choosing the *Bifidobacterium sp.* strains the origin of the isolates was also taken into consideration.

Although we did not isolate any of the *Bifidobacterium* spp., the *B. animalis* was of chicken origin and was chosen for this reason. In this study we isolated 7 *Lactobacillus spp.* from the cecum of an adult hen, and compared their ability to grow in varying concentrations of bile and decreasing pHs with that of 3 commercial isolates. Compared to all other *Lactobacillus* species, *L. fermentum* A demonstrated the ability to grow under the lowest pH and in the presence of the highest concentration of bile.

Interestingly, this isolate was better suited to growth under these conditions than another *L. fermentum* isolated from the same chicken. Although we did not verify that these two isolates were capable of colonizing any regions of the intestinal tract, it has been shown that it is possible for *Lactobacillus* to colonize the intestinal tract of young chicks, even in the absence of any real affinity for intestinal epithelial cells (Rada *et al.*, 1995). In the absence of any direct evidence of intestinal colonization by either of our isolates, our administration of both probiotic strains attempted to ensure maximum intestinal presence by providing gavaging daily for 7 days. Although the gavaging titre was several orders of magnitude below the overall total number of microbes in the gut environment, it was similar to the total numbers of endogenous *Lactobacillus* and *Bifidobacterium* spp. found in the cecum, and thus ensured the number of probiotic probiotic organisms to exceed what would be normally present.

Despite the demonstrated *in vitro* ability to survive both the pH and bile in the proximal intestine, neither *L. fermentum* nor *B. animalis* were capable of inhibiting the intestinal colonization by *S. enteritidis* and *C. jejuni* in either the ileum or cecum. Thus, survivability is not the only parameter that must be addressed when choosing probiotic strains. Other parameters that have been examined include the *in vitro* ability to inhibit the growth of other intestinal microorganisms and the ability of the probiotic strain to attach to the intestinal epithelial surface (Garriga *et al*., 1998; Mishra and Prasad, 2005), however survivability is often the only measurable parameter *in vivo* (Garriga *et al.*, 1998). In both experiments *L. fermentum* did not reduce intestinal colonization by *S. enteritidis*, however the actual number birds which were *S. enteritidis* positive in the liver and spleen were reduced (6/18 and 8/18 in trial 1 and 6/19 and 7/19 in trial 2 for

the liver and spleen, compared to the negative control groups which had a total of 9/19 and 12/19 in trial 1 and 9/20 and 10/20 in trial 2, respectively). Together this would suggest that *L. fermentum* administration did appear to inhibit the intestinal translocation of *S. enteritidis*. The ability of both *Lactobacillus* and *Bifidobacterium* spp. to reduce translocation of *Salmonella* spp. has been previously reported both *in vitro* and *in vivo* (Gill *et al*., 2001; Hudault *et al*., 1997; Shu *et al*., 2000), however in our challenge model *B. animalis* was not able to inhibit the translocation of *S. enteritidis*. The inhibition of pathogen translocation may have been the result of at least two mechanisms; 1) bacterial interference whereby the probiotic organism restricts or blocks the ability of the pathogen to attach to epithelial cells (Patterson and Burkholder, 2003) or 2) the immunostimulatory effect of *Lactobacillus* spp. (Erickson and Hubbard, 2000; Perdigon *et al*., 2001). It has previously been shown that *L. fermentum* is able to attach to epithelial cells (Reid *et al.*, 1993), which might limit the ability of *S. enteritidis* to attach to intestinal epithelial cells. Thus, although *L. fermentum* might not reduce the pathogen load, it might play an important role in protection against pathogens which have a contact-dependant, or Type-III secretion system (Wigley *et al.*, 2002) such as *Salmonella* spp..

In addition to being able to simply colonize the intestinal tract, it has been hypothesized that *Lactobacillus* and *Bifidobacterium* spp. out-compete other intestinal microorganisms by producing antimicrobial compounds (Fooks and Gibson, 2002a, 2002; Gibson and Fuller, 2000; Patterson and Burkholder, 2003; Servin, 2004). In this study, however, we did not assess the ability of either of the isolates to inhibit other microorganisms *in vitro* or *in vivo*. The production of antimicrobial fermentation end-

products from fructooligosaccarides is a major mechanism by which *Lactobacillus* and *Bifidobacterium* spp. inhibit the growth of pathogenic microorganisms (Vandenbergh, 1985), however our diets did not include the addition of any fructooligosaccharides. This might have contributed to the inability of either strain to inhibit intestinal colonization by *S. enteritidis* and *C. jejuni*, however this remains speculative.

Additional factors which might have contributed to the inability of either *L. fermentum* or *B. animalis* to reduce intestinal colonization by *S. enteritidis* or *C. jejuni* include our infection model. To ensure a large, measurable, and consistent level of pathogen colonization, our infection model involved the infection of day-of-hatch chicks with a relatively high level of pathogen challenge (0.9 mLs of a ~1.0 X 10^8 CFU/mL resuspended culture of either *S. enteritidis* or *C. jejuni*). Although the administration of pathogen on day-of-hatch is reasonable as this is when birds are the most vulnerable to pathogen colonization, the pathogen titre may have been too high for our probiotic treatment to have any effect regardless of the gavaging titre (0.9 mLs of a ~1.0 X 10^8 CFU/mL), or duration (7 days). Other research has used challenge models which employed pathogen titres both similar to those used in this research (Orndorff *et al.*, 2005), as well as titres considerably less (Al-Tarazi and Alshawabkeh, 2003), and many have shown positive results. Thus, it would be interesting to assess the effect that this same level of probiotic administration might have on different pathogen challenge titres.

These studies suggest that when administered singly neither the *L. fermentum* nor *B. animalis* isolate used in these studies were capable of significantly reducing intestinal colonization by *S. enteritidis* or *C. jejuni* using our challenge model. There was an

indication, however, that *L. fermentum* may be able to reduce intestinal translocation of *S. enteritidis*. In terms of the spread and persistence of pathogens within poulry flocks, inhibition of pathogen translocation could be an important finding and thus further studies need to be undertaken which examine the conditions in which this might be possible. Selecting probiotic strains that are both host-specific and capable of withstanding the harsh intestinal conditions does not appear to ensure both survival and efficacy. Further research must be undertaken which might add additional insight into the factors which contribute to their successful application.

4. THE EFFECT OF HEN-EGG ANTIBODY ADMINISTRATION ON *CAMPYLOBACTER JEJUNI AND SALMONELLA ENTERITIS* COLONIZATION IN THE GASTROINTESTINAL TRACT OF BROILER CHICKENS

4.1. Abstract

Hen-egg antibodies have been shown to be efficacious in reducing pathogen shedding and gastrointestinal disease, however, their application for the reduction of pathogens such as *S. enteritidis* and *C. jejuni* in broiler chickens has not been investigated. In this study we examined the effectiveness of using hen-egg antibodies for the reduction of intestinal colonization by these important pathogens. Laying hens were vaccinated with either *S. enteritidis* or *C. jejuni* and the resulting eggs collected. Egg yolks were pooled, mixed in feed at 0.05% (w/w) or prepared for oral gavage by either water extraction using phosphate-buffered saline, or concentration using ammonium sulphate. The *in vitro* ability of the pathogen-specific antibodies to inhibit pathogen attachment to rat epithelial cells and porcine mucin was demonstrated. In addition, we demonstrated *in vivo* intestinal activity of anti-*S. enteritidis* antibody using day-of-hatch chicks. In separate trials, day-of-hatch broiler chicks were experimentally infected with either *S. enteritidis* or *C. jejuni*, and following challenge, hen-egg antibodies were administered in the feed or via oral gavage with either phosphate

buffered saline extracted (1/10 diluted) or ammonium-sulphate precipitated (10X concentrated) antibody. On days days 4, 7, and 11 jejunum, ileum, and cecum contents were collected and plate counts obtained for the appropriate pathogen. Despite measurable hen-egg antibody activity *in vivo* and *in vitro*, we were unable to demonstrate any significant reduction in the intestinal colonization by either *S. enteritidis* or *C. jejuni.*

4.2. Introduction

Salmonella enteritidis and *Campylobacter jejuni* are the two human pathogens most often associated with poultry meat. A Canadian nation-wide survey revealed that 76.9% of randomly selected chicken broiler flocks were contaminated with *Salmonella* spp. (Poppe *et al.*, 1991), *S. enteritidis* being one of the main species. Another study reported that between 77 and 100% of poultry are carriers of *C. jejuni* (Munroe *et al.*, 1983). During processing these bacteria are often disseminated from live poultry to chicken carcasses and by-products, which are then packaged and sent for retail distribution. A study in the United States revealed that 83% of retail chickens were contaminated with *C. jejuni* (Kinde *et al.*, 1983). Proper handling and hygienic practices at the processing plant level reduces the risk of carcass contamination by these pathogens, however these practices alone have not eliminated them from the food supply. Clearly, an approach that includes their reduction at the farm level, before arrival at the processing plant, could lead to less contamination of meat by intestinal bacteria, and thus increase the safety of poultry meat and poultry meat products.

Modern rearing practices, and their resulting high housing density, facilitates the vertical and horizontal transmission of pathogens within flocks making both *S.*

enteritidis and *C. jejuni* difficult to manage at the farm level. The epidemiology and transmission routes of both these human pathogens is only partially understood which makes intervention strategies difficult to design and implement (Guard-Petter, 2001; Sahin *et al*., 2002). Complicating matters is that both of these bacteria are not usually pathogenic to chickens and there is no apparent pathology or effect on growth and therefore no obvious need for any therapeutic strategies (Boyd *et al*., 2005; Guard-Petter, 2001; Smith *et al*., 2005). Transmission of both *S. enteritidis* and *C. jejuni* can occur horizontally through the contaminated feces of infected birds within the flock, as well as from a number of vectors and fomites including workers and transportation equipment (Ramabu *et al.*, 2004). Recent research has also indicated that vertical transmission from breeder to progeny flocks may also be a source of infection (Sahin *et al*., 2002).

A highly attractive and potentially effective approach for the control of pathogens present in the intestinal tract is the administration of specific neutralizing antibodies in the diet (Berghman *et al*., 2005; Mine and Kovacs-Nolan, 2002). Egg-yolk from hyperimmunized laying hens has been shown to be an abundant and inexpensive source of antibodies (Mine and Kovacs-Nolan, 2002), especially compared to other sources. The use of HEA to control both bacterial and viral intestinal pathogens has been examined in various animal and avian species, however the results have been mixed.

Several studies have reported that HEA specific for *Escherichia coli* protect pigs against challenges with enterotoxigenic *E. coli* (Imberechts *et al*., 1997; Marquardt *et al*., 1999; Wiedemann *et al*., 1991; Yokoyama *et al*., 1992). This protection has been shown to be particularly effective when colonization factors such as attachment

fimbriae were used to hyperimmunize the laying hens. Using purified F4 antigen to generate HEA Marquardt *et al.* (1999) and Wiedemann *et al.* (1991) demonstrated protection against enterotoxigenic $F4^+$ *E. coli* in neonatal and early-weaned piglets. Similarily, using F18 antigen Yokoyama *et al.* (1997) and Imberechts *et al.* (1997) produced protective HEA to treat virulent $F18^+$ *E. coli*. In addition to protecting swine from *E. coli* infections, HEA have also been shown to be effective in reducing mortality in neonatal calves that were experimentally challenged with *Salmonella typhimurium* and *Salmonella dublin* (Yokoyama *et al.*, 1998a). In contrast to these positive results however, Letellier *et al.* (2000) examined the use of HEA to control *Salmonella typhimurium* in swine with results that suggested there was no significant reduction in the infection of experimentally challenged pigs. Fulton *et al*. (2002) reported that ducks administered anti-*Salmonella enteritidis* in drinking water were partially protected against a *S. enteritidis* challenge, however full protection was conferred only with the co-administration of a probiotic. Kobayashi *et al.* (2004) found that that HEA were incapable of eliminating a *Cryptosporidium parvum* infection in experimentally infected mice, and Kassaify and Mine (2004) recently demonstrated that both non-immunized and immunized HEA confers the same level of protection to laying hens challenged with *S. enteritidis*. In addition to their ability to reduce the passage of *S. enteritidis* across the basolateral membrane in human epithelial cells (Sugita-Konishi *et al.*, 2000), HEA have been shown to reduce mortality in rainbow trout from *Yersinia ruckeri* infections (Lee *et al.*, 2000), as well as rotavirus infections in mice (Bartz *et al.*, 1980). The objective of the present study was to evaluate the ability of anti-*S. enteritidis*

and anti-*C. jejuni* specific HEA to reduce the number of these pathogens in the gastrointestinal tract of broiler chickens.

4.3. Materials and Methods

4.3.1. Bacteria and culture conditions

A *S. enteritidis* isolate of chicken origin was obtained from Dr. Manuel Chirino, Dept. of Veterinary Microbiology, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK Canada. The isolate was screened for both Nalidixic Acid (25µg/ml) and Novobiocin resistance (25µg/ml) (Nal^r $Novo^r$), with the resulting isolate used for both antibody production and animal challenge studies. For *C. jejuni* antibody production and animal challenge studies, a Nalidixic Acid resistant (125µg/ml) *Campylobacter jejuni* (Nal^r) isolate was obtained from Dr. Dianne Taylor, Dept. of Microbiology, University of Alberta, Edmonton, AB, Canada. The *Salmonella enteritidis* (Nal^r $Novo^r$) isolate was grown aerobically in Trypticase Soy Broth (Difco) at 37ºC until late log phase (6 hour). For animal challenge studies, the cells were harvested by centrifugation (3640 x g for 15 min at 4ºC), washed twice and resuspended in phosphate buffered saline (PBS, pH 7.2) to a final titre of approximately 1.0×10^8 CFU/mL. For bacterin production, the centrifuged cells were resuspended in PBS containing 0.06% formalin to a final titre of 1.0×10^9 CFU/mL. After resuspension in formalin, the cells were placed at 4°C for 1 week. Following 1 week at 4°C, the cells were centrifuged (3640 x g for 15 min at 4ºC) and resuspended in PBS containing 0.05% sodium azide, stored at 4°C, and used for immunizing laying hens. The *C. jejuni* (Nal^r) isolate was grown on blood agar plates containing 125µg/ml Nalidixic acid, aseptically scraped from plates, washed twice and resuspended in PBS as above. The

resuspended cells (10^8 CFU/mL) were used in animals challenge studies, or formalin killed as above, washed twice, and resuspended in PBS containing 0.05% sodium azide, stored at 4°C, and used for immunizing laying hens.

4.3.2. Antibody Production

A total of 8 commercial White Leghorn chickens were used for the production of antibodies. Four laying hens each were immunized with either the *S. enteritidis* or the *C. jejuni* bacterin produced as described above. The bacterin was emulsified with an equal volume of complete Freund's adjuvant for the primary immunization, and Freund's incomplete adjuvant for all subsequent booster immunizations. The immunization dose was approximately 1.0 x 10^9 CFU of killed *S. enteritidis* or *C. jejuni* in a volume of 1.0 mL equally divided between 2 intramuscular injection sites in the breast muscle. Hens were boosted every two weeks for a total of eight weeks until antibody titres failed to increase in collected eggs (as measured using enzyme-linked immunosorbent assay (ELISA), described below). Once high anti *S. enteritidis* and *C. jejuni* antibody titres had been established, eggs were collected and the yolks were separated, pooled, and stored at -20^{o}C.

4.3.3. Concentration and Preparation of Antibodies

The hen-egg yolk antibodies were purified and concentrated using ammonium sulfate precipitation. Egg yolks were thawed and diluted 1:10 (w/v) using deionized water containing 0.02% Sodium Azide, and mixed well using a homogenizer (Polytron T45/2). The water egg yolk mixture was adjusted to pH 5.4 and placed at 4°C overnight. The next morning the supernatant was decanted and solid ammonium sulfate added to a give a final concentration of 25 % (w/v) and stirred for 30 min at room temperature.

The solution was centrifuged (3640 x g for 30 min at 4°C) with the resulting pellet resuspended in 100 mL PBS per litre of starting material. To the resuspended pellet 25% (w/v) ammonium sulfate was added and stirred for 30 min at room temperature, centrifuged and the resulting pellet resuspended in a minimal amount of PBS. Finally, the resuspended pellet was dialized overnight at 4°C against 2 changes of PBS, and frozen.

4.3.4. Measurement of Antibody Titres

Egg antibody titres were measured using an enzyme-linked immunosorbent assay (ELISA). A 96 well microtitre plate (Immunlon 2, Dynatech Laboratories Inc., Chantilly, VA) was coated overnight at 4°C with either 10^6 CFU of *S. enteritidis* or *C. jejuni* suspended in PBS. The plate was washed 4x with PBS containing 0.05% Tween-20 (PBS-T), after which 100 µL volumes of doubling dilutions of the samples were added. The microtitre plate containing the sample was then incubated at 37°C for 1 h. Following incubation the plate was washed 4x with PBS-T, and 100 µL of a horseradish peroxidase-conjugated mouse anti-chicken IgG antibody (Sigma) diluted 1/1000 with PBS-T was added and the plate was incubated at 37°C for 1 h. The plate was then washed 4x again with PBS-T and incubated for 15 min with 50 µL of a solution consisting of 1 mg/mL ABTS substrate (Boehringer Mannheim Biochemica, Germany). The absorbance of each well was determined at 450 nm using an automated spectophotometer (Molecular Devices, V_{max} Kinetic microplate reader; Molecular Devices, Menlo Park, CA). Egg antibody titres were reported as the highest dilution in which the optical density (OD) was greater than the OD of the mean plus three standard errors (SE) of a series of control wells containing egg yolks obtained from normal

unimmunized hens. Antibody titres in feed and intestinal contents were determined as above with control wells containing PBS instead of normal egg yolk.

4.3.5. Animal Protocols

Experimental protocols were approved by the Animal Care Committee of the University of Saskatchewan and were performed in accordance with the recommendations of the Canadian Council on Animal Care (1993). Experimental birds were housed in heated battery cages and at the Animal Care Facility of the Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK. In each experiment, 30 mixed-sex day-of-hatch Ross 308 broiler chicks (Lilydale Hatchery, Wynyard SK) were used. Upon arrival birds were orally challenged with 0.5 mL of a 1 x 10^9 CFU/mL culture of *S. enteritidis* for trials evaluating the anti-*S. enteritidis* HEA, or 0.5 mL of 1 x 10^8 CFU/mL culture of *C. jejuni* for trials evaluating the anti-*C. jejuni* HEA. Infected birds were subsequently placed into 2 cages, with 15 birds per cage, and provided both water and a non-medicated commercial starter crumble *ad libitum* (Co-op Feeds, Saskatoon, SK). To allow pathogen colonization birds were allowed to rest 3 hours prior to antibody administration.

4.3.5.1. Experiment 1 (evaluation of in-feed administration)

In experiments separately evaluating either the anti-*S. enteritidis* HEA or the anti-*C. jejuni* HEA, anti-*S. enteritidis* or anti-*C. jejuni* hen egg antibody yolk was added to the non-medicated starter crumble and mixed thoroughly to a final concentration of 0.5% (w/w). In each experiment the control group received feed containing egg antibodies from unimmunized laying hens at the same final concentration. To preserve antibody activity, the antibody amended feed was placed at 4ºC until just prior to use. On days 4,

7 and 11 post-challenge, 5 birds from each group were euthanized by cervical dislocation and the intestinal contents from the jejunum, ileum, and cecum collected for enumeration of either *S. enteritidis* or *C. jejuni*.

4.3.5.2. Experiment 2 (evaluation of low-antibody-titre gavage)

In separate experiments evaluating each pathogen-specific HEA, birds in the treatment group were orally gavaged with 0.9 mL of either the non-concentrated anti-*S. enteritidis* HEA or anti-*C. jejuni* HEA, resuspended in PBS (PBS extracted) 3 hours post-infection. The control group received PBS extracted HEA from unimmunized hens. Birds were gavaged daily for 7 consecutive days. On days 4, 7 and 11 post-challenge, 5 birds from each group were euthanized by cervical dislocation and the intestinal contents from the jejunum, ileum, and cecum collected for enumeration of either *S. enteritidis* or *C. jejuni*.

4.3.5.3. Experiment 3 (evaluation of concentrated-antibody gavage)

In separate experiments evaluating each pathogen-specific HEA, birds in the treatment group were orally gavaged with 0.9 mL of the ammonium-sulfate-precipitated anti-*S. enteritidis* HEA (10x concentrated) 3 hours post-infection. The control group received ammonium-sulfate-precipitated HEA from unimmunized hens. Birds were gavaged daily for 7 consecutive days. On days 4, 7 and 11 post-challenge, 5 birds from each group were euthanized by cervical dislocation and the intestinal contents from the jejunum, ileum, and cecum collected for enumeration of either *S. enteritidis* or *C. jejuni*.

4.3.6. Sample collection and pathogen enumeration

In all experiments, the entire contents of the jejunum, ileum, and cecae were

separately removed from each bird. The collected contents from each intestinal location was then mixed thoroughly and a subsample of each was placed in sterile peptone containing 0.5% cysteine hydrochloride and immediately placed on ice. The subsamples were serially diluted and plated in duplicate using an automated spiral plater (Autoplate, Spiral Biotech Inc., Bethesda MD, USA). For the enumeration of *C. jejuni* samples were plated on Modified Preston's Campylobacter Isolation Agar (Oxoid) containing 125µg/mL Nalidixic acid (Sigma) and incubated at 37°C for 48 hours in the appropriate atmospheric conditions (BBL271045 Campy-Pac; Becton Dickinson Microbiology Systems). Enumeration of *S. enteritidis* was done using Brilliant Green Agar (Difco) containing 25 µg/mL of Nalidixic acid and Novobiocin (Sigma), incubated for 16 hours aerobically. Statistical analyses of the $\log_{10}$ transformed CFU was performed using SPSS (v.10.0.5, SPSS Inc, Chicago IL, USA).

4.3.7. *In vitro* adhesion assay

To evaluate the ability of the HEAs to inhibit pathogen attachment to epithelial cells and mucin, an actively growing culture of *S. enteritidis* was harvested, centrifuged and then resuspended in thymidine-free media containing 1% [methyl, 1,2-^{3}H]thymidine (Amersham International, UK) (total activity 10µC/mL) and incubated aerobically at 37ºC for 9 hours. The ^{3}H-labeled *S. enteritidis* culture was washed twice, resuspended in HEPES-Hanks buffer, and incubated in the presence of doubling dilutions of anti-*S. enteritidis* HEA for 1 hour. Unbound antibody was removed by centrifugation and the antibody-coated cells were resuspended in HEPES-Hanks buffer. 1.0 mL of the resuspended cells were then dispensed in triplicate into 24 well sterile sterile tissue culture plates (Nunc) that had been coated with either rat small intestine epithelial cells

grown to confluency (cell line IEL-18, ATTC, Rockwell, Maryland, USA), or 1.0% porcine mucin in PBS. Both the mucin and epithelial cell coated plates were washed twice with HEPES-Hanks buffer prior to the addition of radiolabeled bacteria. The bacteria were incubated in the tissue culture plates for 1 hour at 37°C. For the effect of HEA on the ability of *C. jejuni* to attach to mucin, harvested cells were treated identical to the *S. enteritidis* except incubation was done in a 1L anaerobic jar (Becton Dickinson Microbiology Systems) containing atmospheric conditions for growth of *C. jejuni* (BBL271045 Campy-Pac; Becton Dickinson Microbiology Systems), and the resulting radiolabelled *C. jejuni* were incubated in the mucin coated plates for 3 hours. In all cases, non-attached microbes were removed after 1 hour by rinsing plates twice with HEPES-Hanks buffer, and the attached microbes were removed from the enterocytes and mucin by 24 hour incubation in 5% SDS. 100µL of the contents of each well was recovered, placed in scintillation vials and the radioactivity counted using a Beckman LS6000TA Scintillation Counter.

4.3.8. Specific antibody intestinal activity assay

An enzyme-linked immunosorbent assay (ELISA) was used to measure the specific anti-*S. enteritidis* HEA in the intestinal tract. Four day-of-hatch chicks were orally gavaged with 0.9 mL of the non-concentrated, PBS-extracted anti-*S. enteritidis* HEA, or PBS as a negative control. At 1 and 3 hours post administration, 2 birds from each group were euthanized by cervical dislocation. Intestinal contents were obtained by excising and gently rinsing 3cm sections of the jejunum, ileum, and cecum with 1.0 mL enzyme inhibitor solution, and placed into ependorf centrifuge tubes on ice. The composition of the enzyme inhibitor was as follows; 0.15 M NaCl, 0.005 M NaH_2PO_4-

H_2O, 0.005 M Na_2HPO_4, 0.02% sodium azide, 5 mM EDTA-Na_2, 2 mM phenylmethylsulfonyl fluoride, 10 U/mL aprotinin. The ependorf tubes containing the intestinal washes were then vigorously vortexed, centrifuged at 5,000 x g for 10 min at 4°C and the supernatant removed. 100µL of the supernatant containing the HEA was then dispensed into a 96 well microtitre plate (Immunlon 2, Dynatech Laboratories Inc., Chantilly, VA) that had been coated overnight at 4°C with 10^6 CFU of either *S. enteritidis* in PBS, and washed with PBS buffer containing 0.05% Tween-20 (PBS-T). The microtitre plate containing the intestinal washes was incubated at 37°C for 1 hour, washed with PBS-T, and a horshradish peroxidase-conjugated mouse anti-chicken IgG antibody (Sigma) diluted 1/1000 with PBS-T. The wells were washed with PBS-T and incubated with 50µL of a solution consisting of 1 mg/mL of ABTS substrate (Boehringer Mannheim Biochemica, Germany). The absorbance of each well was determined at 450 nm using an automated spectophotometer (Molecular Devices, V_{max} Kinetic microplate reader; Molecular Devices, Menlo Park, CA). Antibody activity was reported as the mean optical density (OD) readings from two birds, after the subtraction of the OD of the mean of a series of 6 control wells containing PBS as a control.

4.4. Results

The anti-*S.* enteritidis and anti-*C.* jejuni HEA endpoint titres from the hyperimmunized hens plateaued at 1: 256,000 and 1:128,000, respectively. Following overnight extraction using PBS at pH 5.6, the antibody titres were slightly less than 1/10 the titre found in the egg yolks and was determined to be 1:16,000 and 1:8,000. After ammonium sulfate precipitation the HEA titres were both determined to be 1:100,000. The ability of the HEA to inhibit attachment of *S.* enteritidis to intestinal enterocytes

and mucin was evaluated *in vitro* using rat epithelial cells (Figure 4.1) and porcine mucin (Figure 4.2). Compared to the negative control where no antibody was added to the radiolabeled *S. enteritidis*, the anti-*S. enteritidis* HEA was able to inhibit the attachment to the rat epithelial cells at all dilutions. The addition of antibody at all dilutions reduced the CPM from 154 ± 20 for the no antibody control (PBS extracted alone), to 42 ± 2 for the 1:16,000 dilution, 59 ± 21 for the 1:32,000 dilution, 83 ± 15 for the 1:64,000 dilution, 58 ± 14 for the 1:128,000, 52 ± 12 for the 1:256,000, and finally 32 ± 15 for the antibody when diluted to 1:512,000. The addition of anti-*S. enteritidis* HEA to the radiolabeled *S. enteritidis* however, appeared to increase the ability of *S. enteritidis* to attach to mucin. Compared to the no antibody control with CPM of 556 ± 49, the addition of the purified, non-concentrated HEA resulted in an increase to 1850 ± 428. As the HEA was diluted the ability of *S. enteritidis* to become associated with the mucin also appeared to diminish.

The addition of anti-*C. jejuni* HEA to *C. jejuni,* however, resulted in a decrease in the ability of the pathogen to attach to the mucin *in vitro* (Figure 4.3). In the absence of antibody, the radiolabeled *C. jejuni* alone resulted in a CPM of 314 ± 69, whereas incubation in the presence of doubling dilutions of pathogen-specific HEA from 1:16,000 to 1:256,000 resulted in a reduction in the CPM.

When administered to day old chicks, the HEA was shown to be active in the intestinal tract up to 3 hours post administration (Figure 4.4). The mean ODs from the intestinal washes obtained from birds euthanized at 1 and 3 hours after oral administration of the PBS extracted anti-*S. enteritidis* antibody resulted in detectable activity above background at both time points and in all locations. At 1 hour post

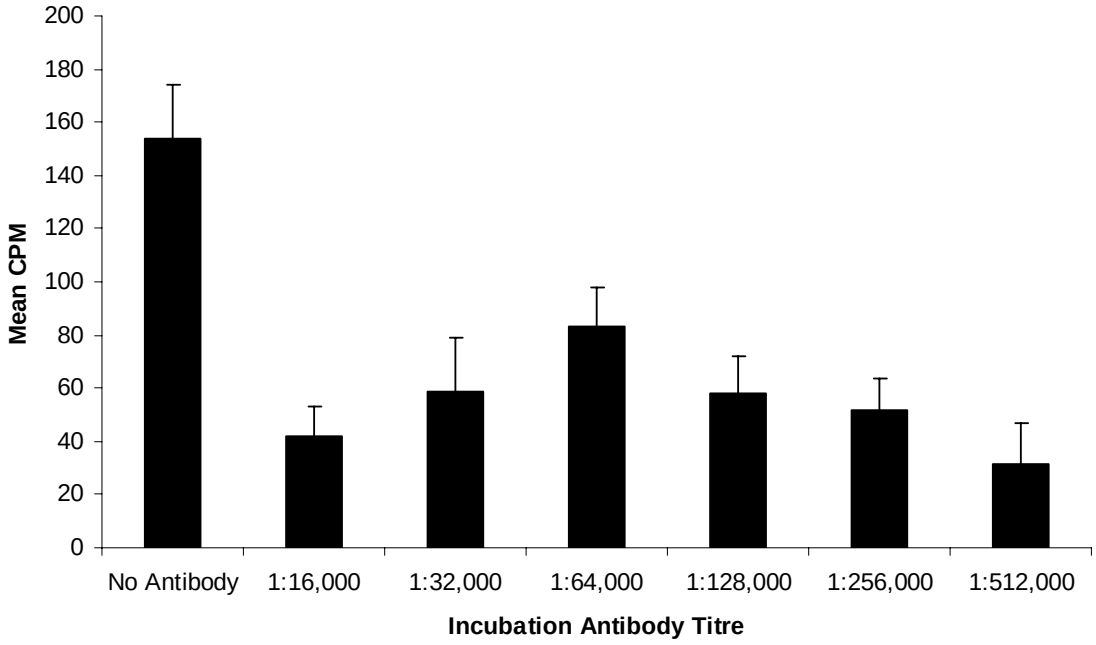

Figure 4.1. ^{3}H-labeled *S. enteritidis* bound to rat epithelial cell line IEL-6 after incubation for one hour in wells containing anti-*S. enteritidis* HEA. Bars are the mean CPM ± standard deviation of 3 wells.

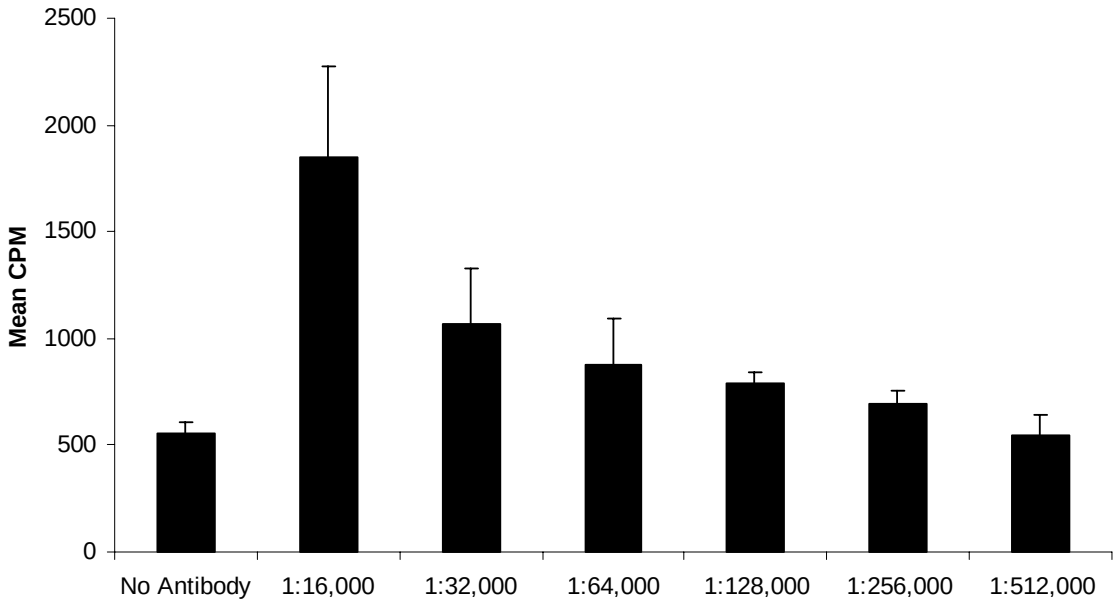

Figure 4.2. ^{3}H-labeled *S. enteritidis* bound to porcine mucin after incubation for one hour in wells containing anti-*S. enteritidis* HEA. Bars are the mean CPM ± standard deviation of 3 wells.

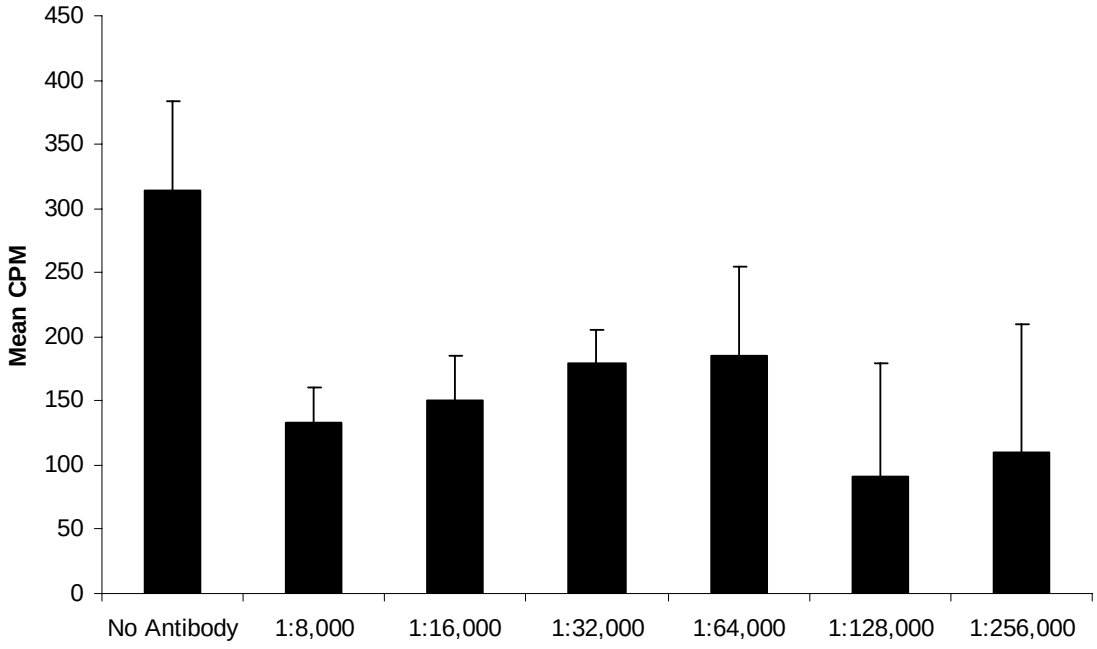

Figure 4.3. ^{3}H-labeled *C. jejuni* bound to porcine mucin after incubation for one hour in wells containing anti- *C. jejuni* HEA. Bars are the mean CPM ± standard deviation of 3 wells.

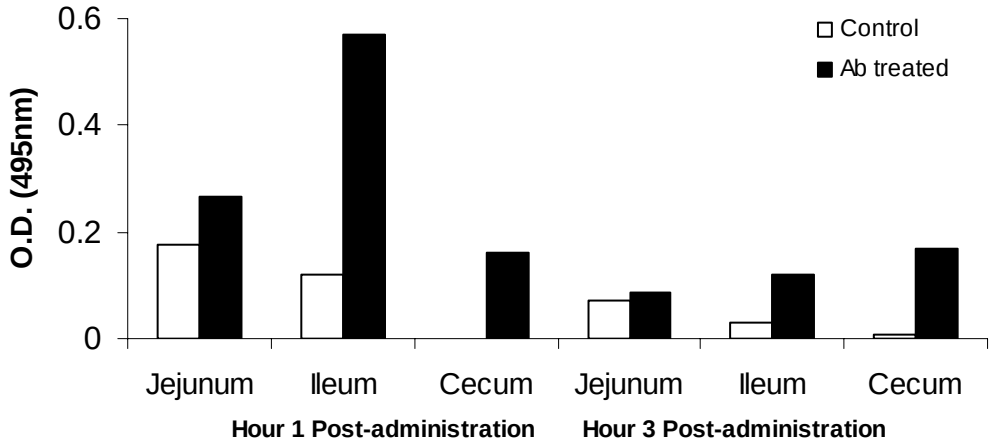

Figure 4.4. Relative intestinal HEA activity in day-of-hatch broiler chicks orally gavaged with anti-*S. enteritidis* PBS extracted antibody at 1 and 3 hours post-gavage. The Bars at each intestinal location represent the mean O.D. value of 2 birds.

administration, the relative ODs were 0.265, 0.571, and 0.160 for the jejunum, ileum, and cecum, compared to 0.175, 0.121, and 0.000 for the PBS gavaged negative control,respectively. At 3 hours post-administration, detectable antibody was observed, albeit at lower levels than the previous sampling time. Compared to the OD values obtained from birds administered PBS of 0.071, 0.030, and 0.006 for the jejunum, ileum, and cecum, birds administered the pathogen specific HEA antibody had ODs of 0.085, 0.120, 0.168, respectively.

Despite measurable antibody activity of the HEA in the jejunum, ileum, and cecum of chicks, the administration of anti-*S. enteritidis* and anti-*C. jejuni* HEA in the diet (Figures 4.5 and 4.6), or gavaged at either the non-concentrated (Figures 4.7 and 4.8) or concentrated titres (Figures 4.9 and 4.10), did not significantly reduce ($P < 0.05$) the level of intestinal colonization by either pathogen. This observation was true for any time point or intestinal location, while the observed level of colonization of both *S. enteritidis* and *C. jejuni* was consistent with colonization levels observed using this challenge model in previous trials.

4.5. Discussion

Immunization of laying hens resulted in antibody titres that were consistent with, or exceeded titres obtained by other researchers (Marquardt *et al.*, 1999). The PBS precipitated HEA resulted in the lowest titre of 1:16,000, however even at this titre there was measurable antibody activity when experimental day-of-hatch birds were orally gavaged. Measured by ELISA, the *in vivo* passage of antibody was confirmed (Figure 4.4) with continued functionality of the antibody as it transited the intestinal tract. Although the highest level of activity was shown to be in the ileum after 1 hour, activity

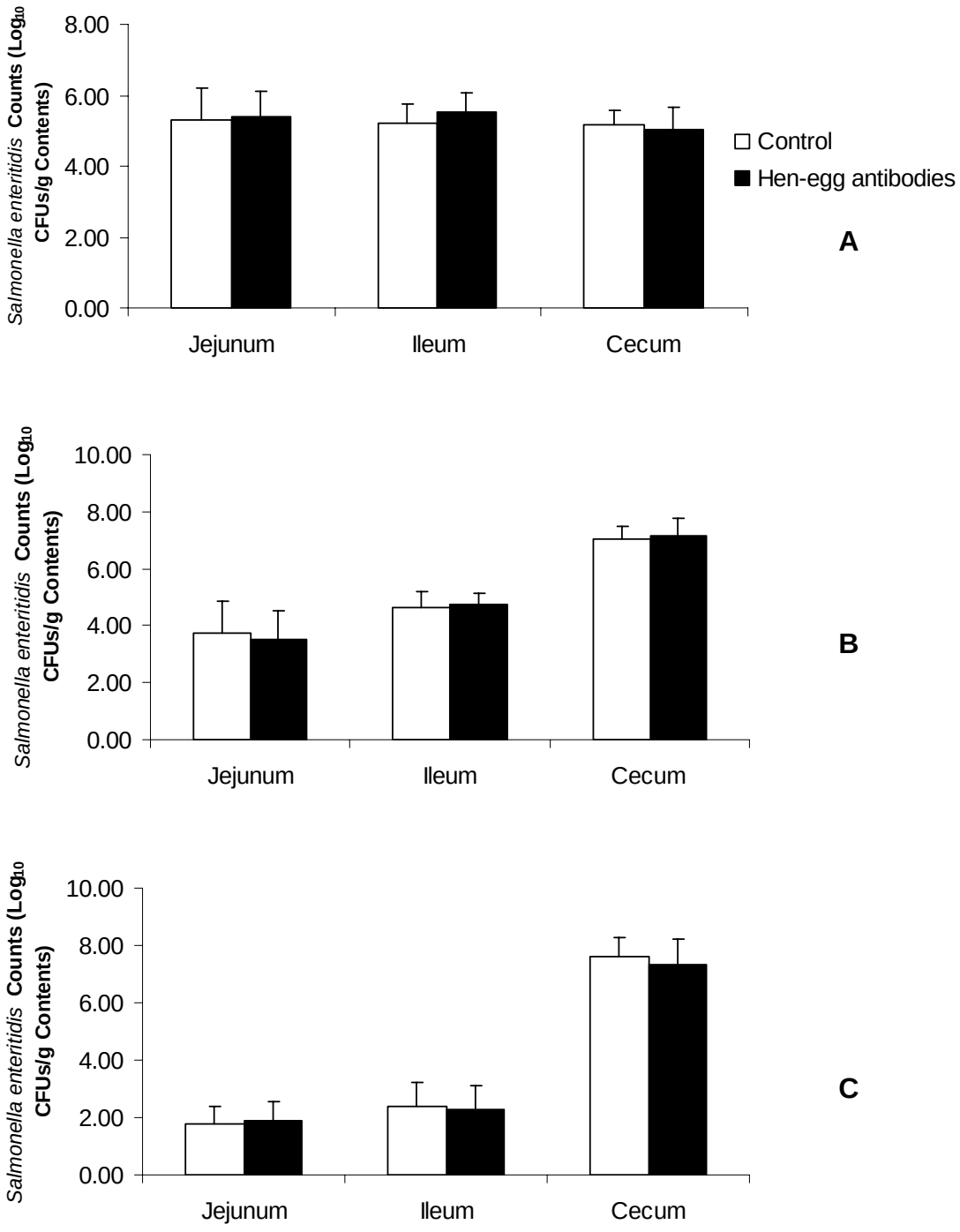

Figure 4.5. Intestinal counts on days 4 (A), 7 (B), and 11 (C) of broiler chicks challenged with *S. enteritidis* and fed anti-*S. enteritidis* HEA amended feed. Bars represent mean ± standard deviation (N=5).

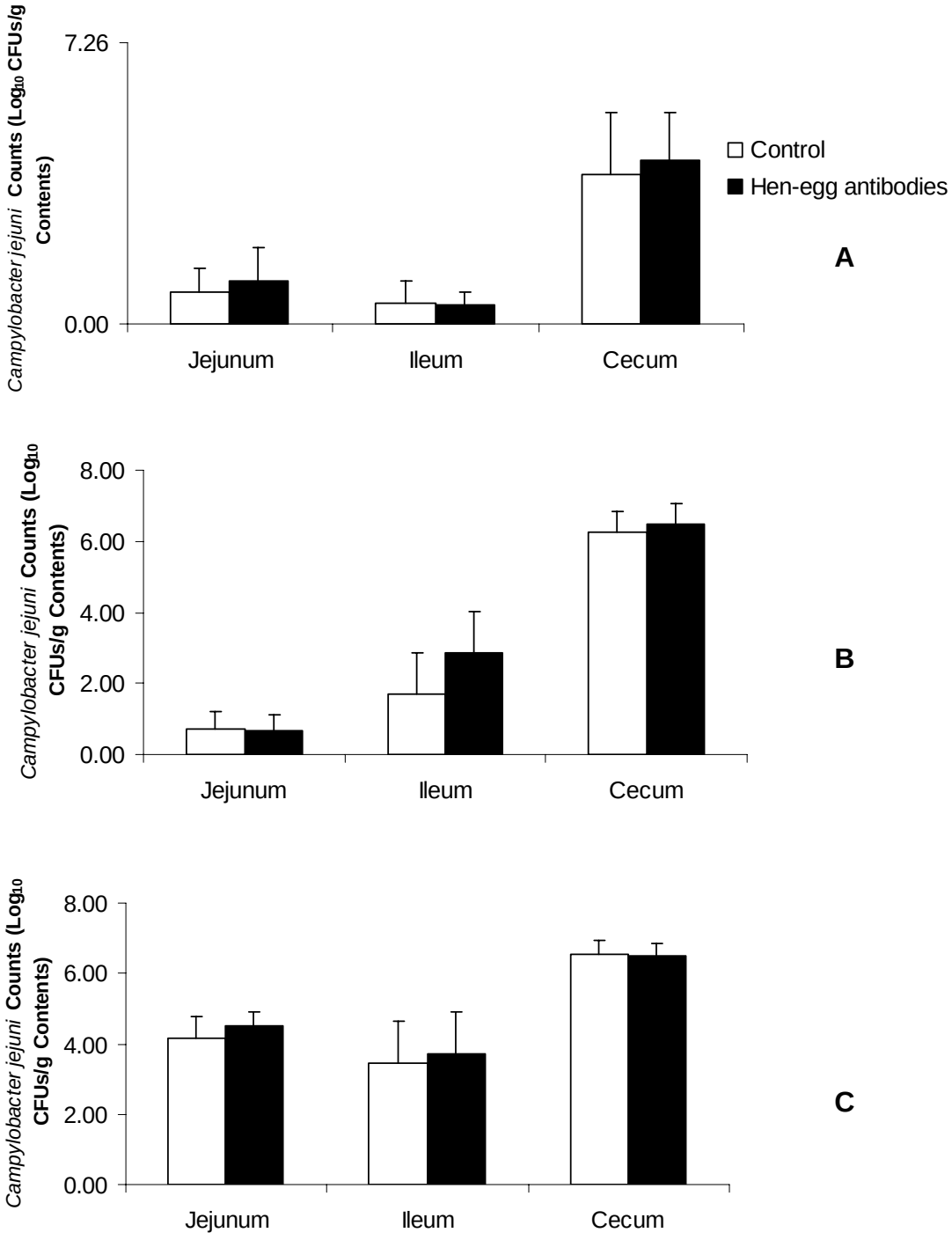

Figure 4.6. Intestinal counts on days 4 (A), 7 (B), and 11 (C) of broiler chicks challenged with *C. jejuni* and fed anti-*C. jejuni* HEA amended feed. Bars represent mean ± standard deviation (N=5).

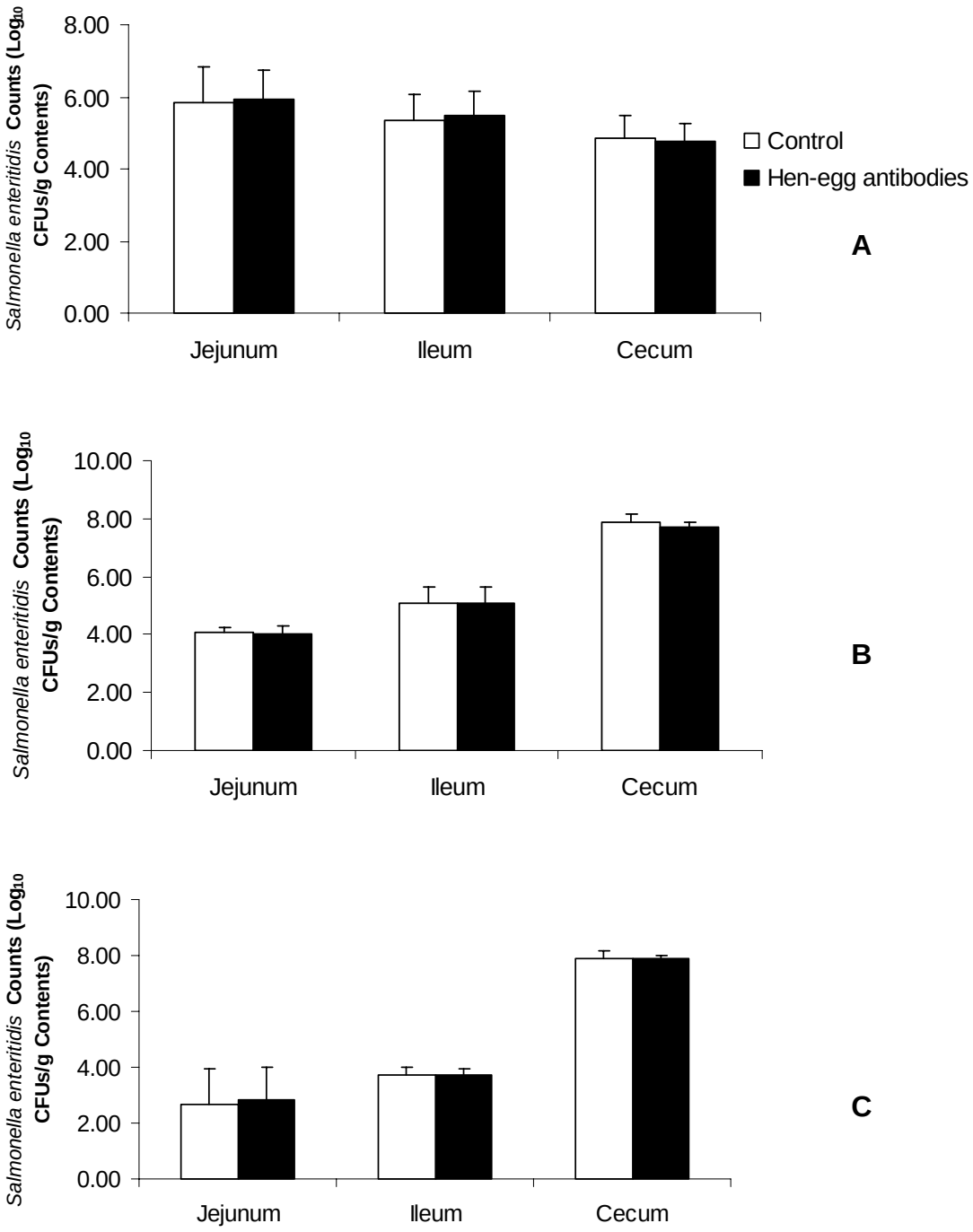

Figure 4.7. Intestinal counts on days 4 (A), 7 (B), and 11 (C) of broiler chicks challenged with *S. enteritidis* and daily orally gavaged with PBS extracted anti-*S. enteritidis* HEA for 7 days. Bars represent mean ± standard deviation (N=5).

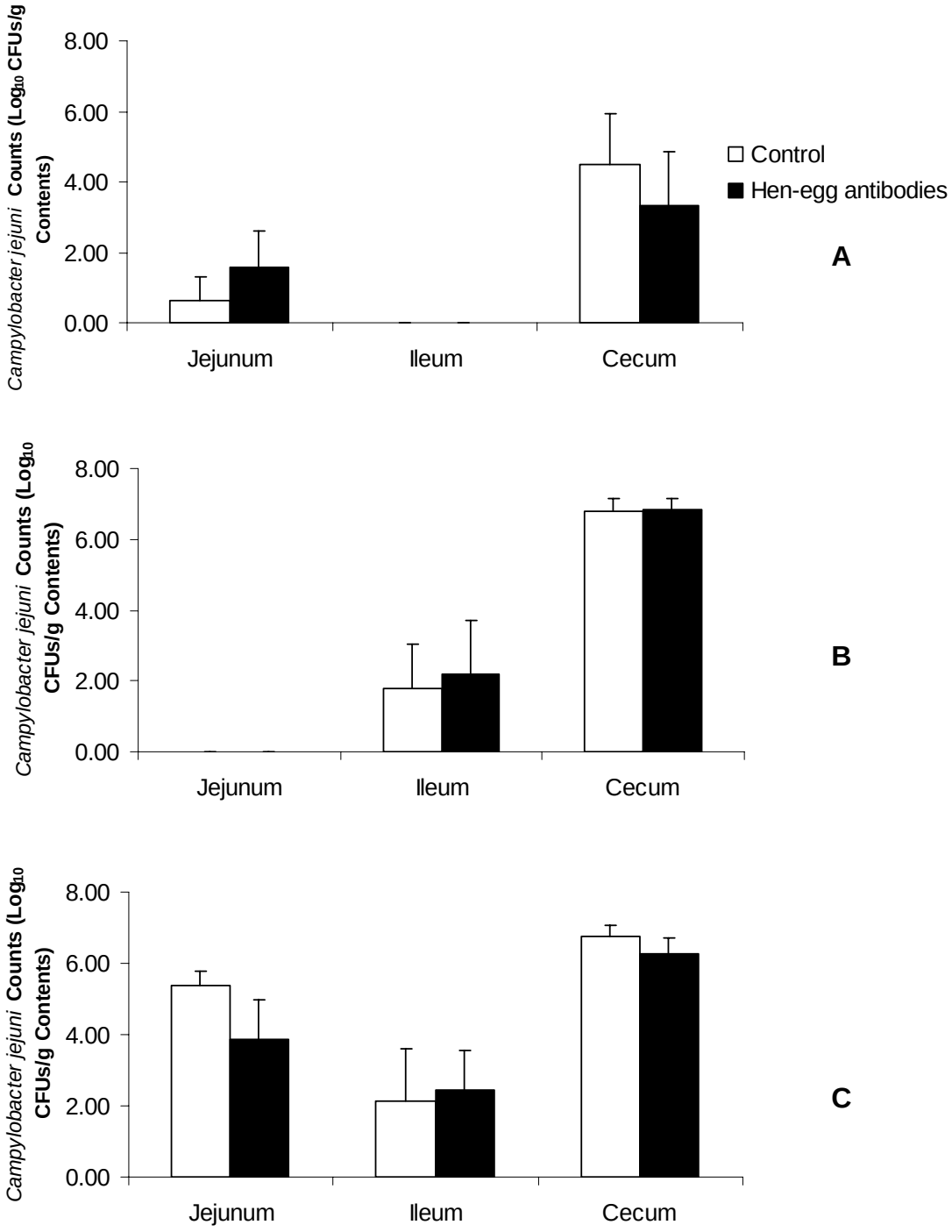

Figure 4.8. Intestinal counts on days 4 (A), 7 (B), and 11 (C) of broiler chicks challenged with *C. jejuni* and daily orally gavaged with PBS extracted anti-*C. jejuni* HEA for 7 days. Bars represent mean ± standard deviation (N=5).

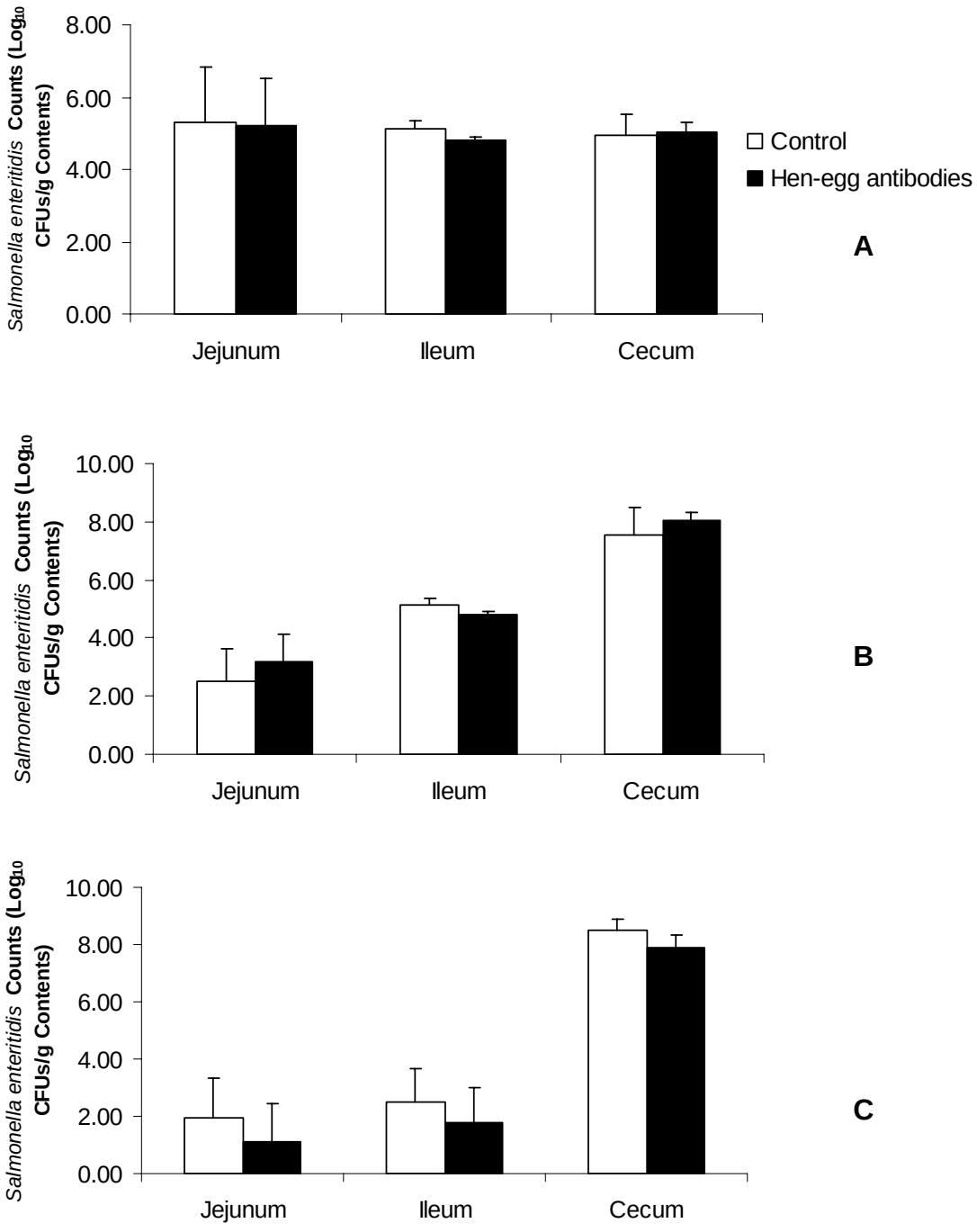

Figure 4.9. Intestinal counts at days 4 (A), 7 (B), and 11 (C) of broiler chicks challenged with *S. enteritidis* and daily orally gavaged with ammonium sulfate precipitated anti-*S. enteritidis* HEA for 7 days. Bars represent mean ± standard deviation (N=5).

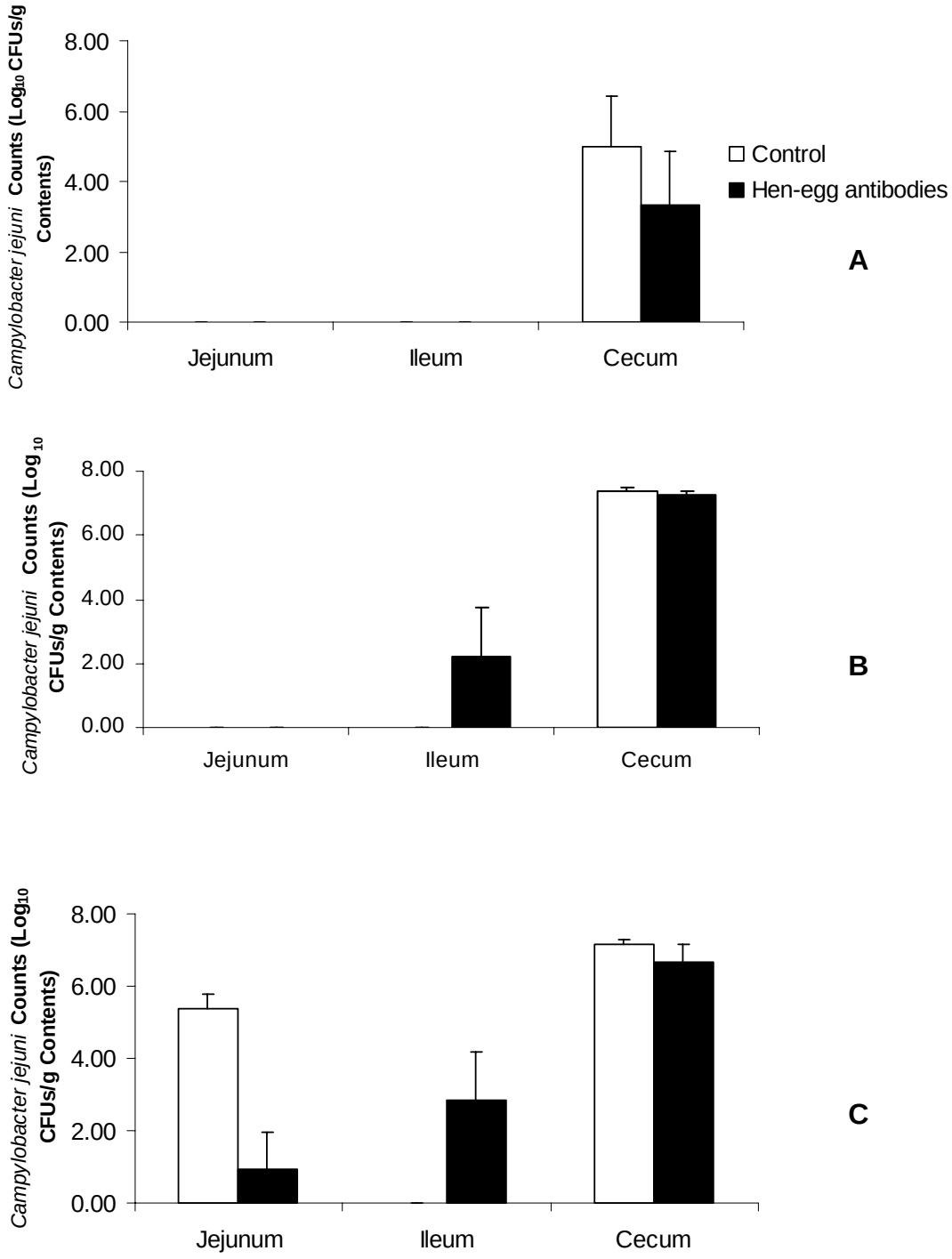

Figure 4.10. Intestinal counts on days 4 (A), 7 (B), and 11 (C) of broiler chicks challenged with *C. jejuni* and daily orally gavaged with ammonium sulfate precipitated anti- *C. jejuni* HEA for 7 days. Bars represent mean ± standard deviation (N=5).

was still present after 3 hours. Interestingly, there was measurable antibody activity in the cecum which possesses the highest number of intestinal microorganisms. The observation that administered antibody retains activity in the intestinal tract agrees with results obtained for other animal species including pigs (Yokoyama *et al.*, 1993) and rainbow trout (Lee *et al.*, 2000). As with these other studies, we administered the antibody as a single bolus dose. The dynamics of a single bolus administration would be different than administration of antibody over a longer period of time, as it would be the case if the antibody mixed with feed. The constant administration in feed would result in stable activity in each compartment, which would be required for prophylaxis or therapy. In this trial we administered the antibody to day-of-hatch chicks, however it is unclear if the antibody activity would be similar or reduced in more mature birds with a more developed digestive capacity and intestinal microflora.

By administering hyperimmunized egg yolk in the feed, the extraction of HEA from yolks using PBS, and further concentration using ammonium sulfate, we were able to evaluate 3 different regimes for the administration of each pathogen-specific antibody. To determine if the constant administration of native antibody, with the potentially protective yolk, would be better able to provide protection than orally gavaged HEA, both anti-*C. jejuni* and anti-*S. enteritidis* HEA were mixed in feed at 0.5% (w/w) for experiment 1. In experiments 2 and 3 however, the experimental birds were orally gavaged with the PBS extracted and concentrated HEA, respectively. Regardless of the route of administration, there was no significant ($P < 0.05$) reduction in the intestinal colonization of either *S. enteritidis* or *C. jejuni*, compared to the negative controls.

Despite the negative results obtained *in vivo*, anti-*S. enteriditis* HEA was able to reduce the ability of *S. enteritidis* to colonize rat enterocytes *in vitro*. The observed attachment inhibition might contribute to a shorter infection period in challenged birds, however it is not clear whether intestinal colonization would be affected during this 2 week experimental period. For pathogens with a Type III secretion system such as *Salmonella* spp. (Mota and Cornelis, 2005), colonization of intestinal surfaces may be a contributing factor for translocation and ultimately the persistence of the infection, however the colonization of mucus and intestinal contents, particularly in the ileum and cecae, may occur independently. The incubation of *S. enteritidis* in the presence of anti-*S. enteritidis* HEA did not decrease, but rather increased, the ability of *S. enteritidis* to become associated with porcine mucin (Figure 4.2). The conditions observed in the intestinal tract are more complex than the *in vitro* system using porcine mucin, however, the observed increase in the ability of *S. enteritidis* to become associated with mucin in the presence of anti-*S. enteritidis* HEA may have contributed to the lack of intestinal clearing even in the absence of epithelial surface attachment. Other researchers have reported an increase in the infection rate among *Salmonella*-challenged birds fed anti-*Salmonella* HEA (Fulton *et al.*, 2002). The authors attributed this increased rate of infection to antibody-mediated opsonization of *Salmonella*. In this study we did not examine the translocation of *S. enteritidis* to other organs, but clearly translocation may have contributed to persistent shedding and a lack of any observed reduction regardless of the route of HEA administration.

The *in vitro* attachment of *C. jejuni* to the rat epithelial cells was impossible to demonstrate as the growth conditions for *C. jejuni* was different than those required for

the maintenance of the cell lines. The particular growth requirements for *C. jejuni*, which prefers a solid surface on which to grow, and thus difficult to label with tritiated thymidine, also resulted in considerably lower CPM values for this pathogen compared to *S. enteritidis* (Figure 4.3). However, even with lower CPM values it was possible to demonstrate an effect of HEA on mucin attachment by *C. jejuni*. Whereas incubation with anti-*S. enteritidis* HEA increased ability of *S. enteritidis* to become associated with the mucin *in vitro*, incubation of *C. jejuni* with anti-*C. jejuni* HEA resulted in a decrease in CPMs compared to the negative control. This finding is consistent with research done by others. In addition to forming a protective barrier overlying the intestinal surface, intestinal mucin also supports the growth of a number of intestinal bacteria including both commensal and pathogenic microorganisms such as *C. jejuni* (Fernandez *et al*., 2000; Sylvester *et al*., 1996), and it would appear that the presence of anti-*C. jejuni* HEA reduced the ability of *C. jejuni* to become associated with mucin *in vitro*.

Other researchers have shown negative results using HEA raised against bacterins (Fulton *et al.*, 2002) and also *Cryptosporidium parvum* oocysts (Kobayashi *et al.*, 2004). Kobayashi *et al.* (2004) suggested that using specific oocyst surface proteins may serve to increase the efficacy of HEA. This may be the case as they appear to be most effective when they target specific virulence factors such as attachment fimbria of *E. coli* (Imberechts *et al.*, 1997; Yokoyama *et al.*, 1997), or outer membrane proteins of *Salmonella* spp. (Yokoyama *et al.*, 1998a). In addition to increasing the specificity of antibodies by vaccinating hens with pathogen-specific virulence factors, other researchers have successfully examined using protected antibodies to improve intestinal activity of orally administered HEA (Kovacs-Nolan and Mine, 2005). Although we did

not attempt to protect the antibody, some sort of antibody protection might enable the antibody to transit the crop, stomach, and proximal intestine where conditions are most harsh. This might increase its survivability and ultimately make it more efficacious.

Thus, despite measurable activity of both anti-*S. enteritidis* and anti-*C. Jejuni* HEA *in vitro*, using our challenge model they did not reduce the intestinal colonization of broiler chicks *in vivo.* It can be concluded therefore, that although HEA show promise as a prophylactic strategy for the reduction of pathogenic microorganisms, more work is needed to understand the pathogenic determinants which may result in effective antibody activity *in vivo* and *in vivo,* as well as the intestinal conditions which reduce their efficacy.

5. THE EFFECT OF HEN-EGG ANTIBODIES ON *CLOSTRIDIUM PERFRINGENS* COLONIZATION IN THE GASTROINTESTINAL TRACT OF BROILER CHICKENS

5.1. Abstract

We evaluated the ability of hen-egg antibodies (HEA) to reduce intestinal colonization by *Clostridium perfringens* in broiler chickens. Antibodies against *C. perfringens* or cholera toxin (negative control) were obtained from the eggs of laying hens hyperimmunized using a *C. perfringens* bacterin or cholera toxin. Eggs were collected, pooled, and egg antibodies were concentrated by polyethylene-glycol precipitation. An initial experiment was conducted to determine the *in vivo* activity of the administered antibody along the length of the intestine. Thereafter, two feeding trials were performed to assess the efficacy of feed amended with the egg antibodies in reducing the level of colonization of *C. perfringens* in challenged birds. Antibody activity declined from proximal to distal regions of the intestine but remained detectable in the cecum. In the first experiment there was no significant reduction in the number of *C. perfringens* in the birds fed the diet amended with the anti-*C. perfringens* egg antibody, compared to the birds that received the anti-cholera toxin egg antibody (n = 10), at any of the sampling times. In the second experiment there was a significant decrease in *C. perfringens* intestinal populations 72 hours after treatment (n = 15) as

assessed by culture-based enumeration, but there was no decrease as measured by quantitative PCR based on the *C. perfringens* phospholipase C gene. Intestinal-lesion scores were higher in the birds that received the anti- *C. perfringens* HEA. Our work suggests that administration of HEA did not reduce the level of *C. perfringens* intestinal colonization and conversely might exacerbate necrotic enteritis.

5.2. Introduction

Intestinal pathogens result in economically important losses to poultry producers annually. *Clostridium perfringens*, the causative agent of necrotic enteritis (NE), is one such pathogen. Presently, the incidence of NE is effectively controlled through the use of antibiotic feed additives; however their outright ban in Europe, as well as consumer concerns in North America, has led to an increased interest in alternatives for the control of intestinal pathogens. The use of orally administered antibodies to control both bacterial and viral intestinal pathogens has been examined in various animal and avian species, with mixed results.

Some studies reported that pathogen-specific HEA can confer protection to pigs experimentally challenged with enterotoxigenic *E. coli* (Imberechts *et al*., 1997; Marquardt *et al*., 1999; Wiedemann *et al*., 1991; Yokoyama *et al*., 1997; Yokoyama *et al*., 1992), neonatal calves experimentally challenged with *Salmonella typhimurium* and *Salmonella dublin* (Yokoyama *et al.*, 1998a), rainbow trout from *Yersinia ruckeri* infections (Lee *et al.*, 2000), and rotavirus infections in mice (Bartz *et al.*, 1980). In contrast to these positive results however, Fulton *et al*. (2002) reported that ducks administered anti-*Salmonella enteritidis* antibodies in drinking water were only partially protected against a *S. enteritidis* challenge, and Letellier *et al.* (2000) examined the use

of HEA to control *Salmonella typhimurium* in swine with results that suggested there was no significant reduction in the infection of experimentally challenged pigs. Kobayashi *et al.* (2004) found that that HEA were incapable of eliminating a *Cryptosporidium parvum* infection in experimentally infected mice, and Kassaify and Mine (2004) recently demonstrated that HEA from non-immunized hens confer the same level of protection to laying hens when challenged with *S. enteritidis* as HEA from hens immunized against *S. enteritidis*.

The use of specific HEA to reduce *C. perfringens* colonization in the intestinal tract of broiler chickens, however, has not been fully explored. We evaluated the ability of anti-*C. perfringens* specific HEA to decrease the number of *C. perfringens* cells in the gastrointestinal tract of broiler chickens, and thereby reduce an important predisposing factor for the development of NE.

5.3. Materials and Methods

5.3.1. Bacteria and culture conditions

A *C. perfringens* Type A isolate isolated from a field case of necrotic enteritis was obtained from Dr. Manuel Chirino, Dept. of Veterinary Microbiology, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK., Canada. To ensure maximum viability of cultures the bacteria were grown in Brain Heart Infusion (Difco) anaerobically at 37°C until late log phase. For bacterin production the cells were harvested by centrifugation (3640 x g for 15 min at 4ºC), washed twice in phosphate-buffered saline (PBS, pH 7.2), and resuspended in phosphate-buffered saline containing 0.06% formalin. After resuspension in formalin the cells were placed at 4°C for 1 week prior to use.

5.3.2. Antibody preparation

The hyperimmunization of laying hens was conducted from January to May 2004 in the Poultry Centre at the University of Saskatchewan. Four commercial White Leghorn chickens were immunized with the *C. perfringens* bacterin produced as described above. The bacterin was emulsified with an equal volume of complete Freund's adjuvant for the first immunization, and Freund's incomplete adjuvant for all subsequent boosters. The immunization dose was approximately 1.0 X 10^9 CFU of killed *C. perfringens* in a volume of 1.0 mL equally divided between two injection sites. Another four hens were immunized with 1.0 mL of a cholera toxin (CTX) solution suspended in sterile PBS (10.0 µg/mL). Hens were boosted every 2 weeks for a total of 8 weeks until antibody titres failed to increase in collected eggs (as measured using enzyme-linked immunosorbent assay (ELISA), described below). Once high anti-*C. perfringens* and anti-CTX antibody titres had been established, eggs were collected and the yolks were separated and pooled. The pooled egg yolks were then diluted 1:6 in distilled water and polyethylene glycol (8000 molecular weight) (PEG) was added to give a final concentration of 30 g/kg of PEG. The resulting mixture was stirred at 4°C for 30 min and then centrifuged at 3640 x g for 15 min at 4°C. The supernatant was recovered and PEG was added to give a final concentration of 120 g/Kg. The mixture was then stirred at 4°C for 30 min followed by centrifugation at 3640 x g for 15 min at 4°C. The precipitate was recovered, resuspended in a minimal amount of PBS, and frozen.

5.3.3. Measurement of antibody activity

Egg-antibody titres were measured using an ELISA. Briefly, a 96-well microtitre plate (Immunlon 2, Dynatech Laboratories Inc., Chantilly, VA) was coated overnight at 4°C with either 10^6 CFUs of *C. perfringens* or 10.0 μg/mL CTX suspended in PBS. The plate was washed 4X with PBS containing 0.05% Tween-20 (PBS-T), and 100 μL volumes of doubling dilutions of the samples were added. The microtitre plate containing the sample was then incubated at 37°C for 1 h. Following incubation the plate was washed 4X with PBS-T, and 100 μL of a horseradish peroxidase-conjugated mouse anti-chicken IgG antibody (Sigma) diluted 1/1000 with PBS-T was added and the plate was incubated at 37°C for 1 h. The plate was then washed again with PBS-T and incubated for 15 min with 50 μL of a solution consisting of 1 mg/mL ABTS substrate (Boehringer Mannheim Biochemica, Germany). The absorbance of each well was determined at 450 nm using an automated spectophotometer (Molecular Devices, V_{max} Kinetic microplate reader; Molecular Devices, Menlo Park, CA). Titres were reported as the highest dilution in which the optical density (OD) was greater than the OD of the mean plus three standard errors (SE) of a series of control wells containing egg yolks obtained from normal unimmunized hens. Antibody titres in feed and intestinal contents were determined as above with control wells containing PBS instead of normal egg yolk.

5.3.4. Experimental diets

A high-protein diet (Table 5.1) previously shown to encourage intestinal colonization by *C. perfringens* was used (Drew *et al*., 2004; Wilkie *et al*., 2005). The PEG-precipitated anti- *C. perfringens* or anti-CTX HEA was added to the feed at 0.05

Table 5.1. Experimental diet used for all birds from days 14-28[1].

Ingredient	g kg^{-1}
Fish meal	329.4
Corn	411.6
Wheat	220.0
Limestone	16.0
Canola oil	17.0
Choline chloride	1.0
Vitamin/Mineral premix[2]	5.0

Footnote to Table 5.1

[1]Diet was formulated to contain 315 g/kg crude protein and meet NRC requirements for broiler chickens.

[2]Supplied per kilogram of diet: vitamin A, 3.3 mg; cholecalciferol, 55 μg; vitamin E, 30 mg; vitamin K, 0.5 mg; vitamin B_{12}; 0.02 mg; thiamine, 1.5 mg; riboflavin, 6 mg; folic acid, 0.6 mg; biotin, 0.15 mg; niacin, 60 mg; pyridoxine, 5 mg; 400 mg; chloride, 788 mg; sodium, 511 mg; iron, 80 mg; manganese, 21.8 mg; selenium, 0.1 mg; iodine, 0.35 mg; zinc, 100 mg.

and 0.065% (v/w) respectively, which provided the same amount of additional protein. The protein concentrations of the PEG precipitated anti- *C. perfringens* and anti-CTX HEA were determined using the Leco FP-528 Protein Analyzer (Leco Corporation St. Joseph MI, U.S.A.) and were 3.44 and 2.65% protein, respectively. Thus, the exogenous protein that was added to both diets from the HEA was 1.7 g/kg. For incorporation into feed, the HEA preparation was sprayed on the feed while mixing. The HEA feed was then stored at 4°C and removed immediately prior to use.

5.3.5. Birds and infection

Two *in vivo* experiments were carried out using day-of-hatch Ross 308 broiler chicks between September 2004 until January 2005 at the Animal Care Facility of the Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon SK, Canada. Experimental protocols were approved by the Animal Care Committee of the University of Saskatchewan and were performed in accordance with the recommendations of the Canadian Council on Animal Care as specified in the Guide to the Care and Use of Experimental Animals (Canadian Council on Animal Care, 1993). In both *in vivo* experiments 1 and 2, birds were challenged with the *C. perfringens* isolate described above. Based on results obtained from previous trials, our challenge model consists of orally gavaging chicks at day-of-hatch with 0.5 mL, and then again for 7 consecutive days from days 14 to 21 with 1.0 mL, of a late log phase culture (10^8 CFU/mL). This infection protocol has proven to result in experimentally infected birds which are consistently and uniformly colonized with *C. perfringens* with similar, albeit less severe, mortality and morbidity typically observed in field cases of NE (Wilkie *et al.*, 2005). After initial gavage, birds were placed into 1 of 2 battery cages, and provided

a nonmedicated commercial starter crumble *ad libitum* (Co-op Feeds, Saskatoon, SK). At 14 days after hatch, the birds were divided into groups of 15 birds per cage, and fed the non-HEA amended experimental diet. This diet was fed to all birds *ad libitum* until day 28. The birds were orally gavaged daily, with 1.0 mL of a *C. perfringens* culture from days 14 through 21 as described above. On day 28 half of the groups were fed the diet that had been amended with anti-CTX, while the other half the diet amended with the anti-*C. perfringens* HEA. At 0, 24, and 72 hours after switching to the HEA-diets, five birds from each cage were euthanized by cervical dislocation and samples of ileal and cecal digesta were taken for the enumeration of *C. perfringens.* In experiment 1, a total of 60 chicks were used for a total of 4 groups of 15 birds each. To increase the statistical power, in experiment 2 a total of 90 chicks were used for a total of 6 groups of 15 birds each. In experiment 2 the entire excised intestinal tracts of the birds were removed and examined macroscopically for the presence of lesions. A lesion score was assigned, via a blind study, for each bird based on a scale from 0 to 4 (0 = normal; 1 = 1 to 5 small red petechiae; 2 > 5 small red petechiae; 3 = presence of focal necrotic lesions; and 4 = presence of patches of necrosis (1-2 cm long)). Micrographs of caecal-tonsil cross-sections showing hemorrhagic lesions were obtained using 6-µm-thick hemotoxylin- and eosin-stained, formalin-fixed tissue using a Axiostar *plus* Zeiss microscope (Carl Zeiss Vision GmbH, Germany) fitted with Axiocam MRc camera.

5.3.6. In vivo antibody activity assay

The anti-CTX antibody titres present along the length of the intestinal tract were measured using 10 Ross 308 broiler chickens at 28 days of age. Birds were raised as above but were not challenged with *C. perfringens*. The HEA-amended experimental

diet was fed *ad libitum* for 48 hours and the birds were then euthanised by cervical dislocation and the entire gastrointestinal tract of the chicken was excised. Intestinal contents were collected from each of the crop, duodenum, jejunum, ileum, and cecum. A small subsample from each of the sections was diluted 1:10 in an enzyme inhibitor solution and immediately placed on ice. The composition of the enzyme inhibitor was as follows; 0.15 M NaCl, 0.005 M NaH_2PO_4-H_2O, 0.005 M Na_2HPO_4, 0.02% sodium azide, 5 mM EDTA-Na_2, 2 mM PMSF, 10 U/mL aprotinin. The diet and intestinal samples were then diluted in a microtitre plate using doubling dilutions of PBS, and the antibody activity determined by ELISA as described above.

5.3.7. Sample collection and pathogen enumeration

In both experiment 1 and 2, the entire contents of the ileum from Meckel's diverticulum to the ileal-cecal junction, and the contents of both cecae were carefully removed from each bird. The collected contents were then mixed thoroughly and a subsample of each was placed in sterile peptone containing 0.5% cysteine hydrochloride and immediately placed on ice. For the enumeration of *C. perfringens* the subsamples were serially diluted and plated in duplicate using an automated spiral plater (Autoplate, Spiral Biotech Inc., Bethesda MD, USA) on BBL™ Blood Agar Base (Becton, Dickinson and Co., Sparks, MD, USA) containing 5.0% sheep blood and 0.01% Neomycin Sulfate (BA^{neo+}) (The Upjohn Company, Orangeville, ON, Canada) The BA^{neo+} plates were incubated anaerobically for 24 hours at 37°C. Colonies exhibiting a double zone of hemolysis on the BA^{neo+} were counted as *C. perfringens*. Several presumptive colonies were picked, Gram stained, and examined microscopically to verify them as *C. perfringens*.

5.3.8. DNA extraction and molecular enumeration of *C. perfringens* in ileal digesta

Samples (0.25g) of ileal contents were placed in a bead beating tube (Mo-Bio Laboratories, Solano Beach, CA) along with RNase A (75 μg), lysozyme (750 μg), and proteinase K (400 μg) in 0.6 ml of a buffer containing Triton X-100 (0.5%) and Tween 80 (0.5%) (Qiagen genomic DNA buffer kit). Guanidine-HCl (675 mM) and Tween 20 (4%) were then added and the samples were incubated at 50°C for 30 min. The samples were subjected to one freeze-thaw cycle at -70°C/25°C, then placed in a bead beater (Bio101 ThermoSavant FP120) and shaken for 20 sec (setting 5) in the presence of 1 vol of 25:24:1 phenol:chloroform:isoamyl alcohol (Sigma). The tubes were centrifuged at 13,000 g for 15 min, the aqueous phase was placed in a new tube, and nucleic acids were precipitated with 1 volume isopropanol. The pellet was washed once with 70% ethanol, dried, and dissolved in 0.1 ml of TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0).

PCR primers (5'- CTGGGGTATCAACTAAAGTCTACGC-3' and 5'-CCTTTGCTGCATAATCCCAATC-3') were designed to amplify a 693 base pair (bp) fragment (PLC-A) of the *C. perfringens* alpha toxin gene (GenBank accession number X17300). These primers (500 nM each) were used in a PCR containing *C. perfringens* genomic DNA (40 ng), 1.5 mM $MgCl_2$, 0.2 mM each dNTP, and 1 U Taq DNA polymerase (Invitrogen) under the following conditions: 94°C, 4 min followed immediately by 40 cycles of 94°C, 1 min; 55°C, 1 min; 72°C, 1 min. The PCR product was purified on an agarose gel and incubated with 1 U Taq polymerase (Invitrogen) and 0.2 mM each dNTP at 72°C for 15 min, then purified using a QiaQuick PCR

purification column (Qiagen). Purified PCR products were ligated into pGEM-T Easy (Promega) overnight at 4°C and the ligation mixture was used to transform *E. coli* JM109 (Promega). A single insert-containing clone was retrieved and used as a PCR standard as described below.

A second set of alpha toxin-specific PCR primers was designed for molecular enumeration of *C. perfringens* using real-time quantitative PCR (qPCR). These primers (5'- GAAGCTATGCACTATTTTGGAGAT-3' and 5'-ATACTGTTCTTTCCTTTCTTCTGC-3') amplified a 120-bp fragment (PLC-B) of the *C. perfringens* alpha toxin gene that was nested within the larger cloned PLC-A fragment. To enumerate *C. perfringens* in ileal digesta samples, we first optimized the dilution of the DNA extracts to mitigate the effects of PCR inhibition (Dumonceaux *et al.*, 2005) . DNA extracts were diluted 1:16 in TE buffer (10 mM Tris-Cl pH 8.0, 1 mM EDTA) and used in a qPCR assay along with standards consisting of known amounts of PLC-A plasmid DNA. Plasmid standards were expressed as copy number per qPCR assay, which was calculated as the weight of plasmid DNA in each standard divided by the plasmid molecular weight (2.4×10^6 g/mol based on a plasmid size of 3697 bp). qPCR assays used Platinum® SYBR Green Quantitative PCR SuperMix-UDG (Invitrogen) and included 3 mM $MgCl_2$ and 500 nM each primer. Amplifications were preceeded by the following steps: 50°C, 2 min (UDG activation), then 95°C, 3 min (well factor collection); this was followed immediately by 40 cycles of 94°C, 30 sec; 55°C, 30 sec; 72°C, 30 sec. Data collection was set at the extension step. Amplifications were performed using an iCycler (BioRad). qPCR counts were converted to genomes detected/g digesta based on the weight of digesta extracted and the dilution factors.

5.3.9. Determination of Phospholipase C activity in intestinal contents

The alpha toxin activity from the intestinal contents of birds euthanized at hour 72 of experiment 2 was investigated using Amplex Red PC-PLC assay kit (Molecular Probes, Eugene OR, USA) according to the manufacturer's instructions. Briefly, 1.0 g of odd-numbered ileal samples from each group (six birds in each of the anti-*C. perfringens* and anti-CTX groups) were diluted 1:1 in the enzyme-inhibitor solution described above. Samples were then vortexed vigorously, centrifuged at 10,000g for 3 min, decanted and the supernatant placed on ice. The supernatant from each sample was then diluted in the buffer provided by the manufacturer to a final dilution of 1/320, and analyzed in duplicate. Phospholipase C concentration was determined by comparing the unknowns, minus the value obtained from a set of duplicate negative controls, against a standard curve obtained from known concentrations of a positive control provided by the manufacturer. The manufacturer stated lower limit of sensitivity of this assay was 0.2mU/mL.

5.3.10. In vitro HEA mediated agglutination

To evaluate HEA-mediated agglutination, 3.3 X 10^8 CFUs/mL *C. perfringens* was incubated with anti- *C. perfringens* and anti-CTX HEA at an endpoint titre of 5.71 for 15 min at room temperature. The incubated solutions were placed on glass slides and agglutination visually evaluated under 400X magnification on a Axiostar *plus* Zeiss microscope (Carl Zeiss Vision GmbH, Germany) fitted with Axiocam MRc camera. The solutions were also plated on BA^{neo+} plates for enumeration of *C. perfringens*.

5.3.11. Statistical analysis

Individual birds were considered the experimental unit in both experiments. Bacterial numbers at each anatomical site were log transformed to normalize them prior to analysis. Statistical analyses of bacterial populations, *in vivo* antibody titres, and phospholipase C concentrations were done using a one-way analysis of variance with diet or anatomical location as the independent variables (SPSS v.13.0, SPSS Inc, Chicago IL, USA). Treatment effects were considered significant when $P < 0.05$. Lesion scores were analyzed using a two-tailed Mann-Whitney U test. Differences between medians were considered significant when $P < 0.05$.

5.4. Results

After immunizing laying hens repeatedly, the pooled anti-*C. perfringens* and anti-Ctx antibody titres were determined to be 1:256,000 and 1:512,000, respectively. Following PEG precipitation the anti-*C. perfringens* and anti-Ctx titres were both determined to be 1:1,024,000. Anti-*C. perfringens* and anti-Ctx titres in feed were 1:60,000 and 1:72,000, respectively. This difference was due to the differences in the amount of total protein in each concentrated HEA. Balancing the amount of added protein resulted in differences in the amount of specific antibodies that were added to each diet. Using the anti-CTX amended feed, measurable antibody activity *in vivo* was observed (Figure 5.1). Except for the crop and duodenum, there was a significant ($P < 0.05$) decrease in the antibody activity moving proximal to distal within the intestine.

In experiment 1 there were no significant differences in the number of *C. perfringens* found in either the cecum or ileum of birds receiving the anti- *C. perfringens* HEA compared to the birds receiving the anti-Ctx HEA at hour 0, 24 or 72

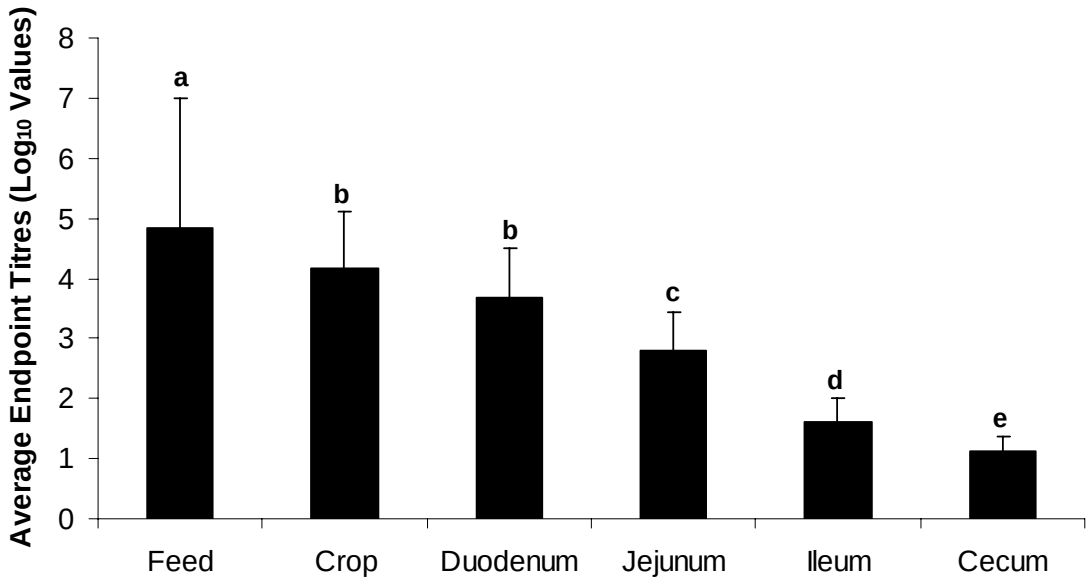

Figure 5.1. The $\log_{10}$ of the inverse endpoint titres of anti-cholera toxin hen-egg antibody in feed and five intestinal locations from experimental chickens after 48 hours of consuming HEA-amended feed. Bars represent the mean ± SEM (n = 10). ^{abcde}Bars with different superscripts are significantly different ($P < 0.05$).

(Figure 5.2). In experiment 2, there was a significantly lower number of *C. perfringens* found in both the ileum and cecum of the birds receiving the anti- *C. perfringens* HEA ($P < 0.05$) at hour 72, however at all other sampling times, there were no significant differences between treatment groups (Figure 5.3). At this same sampling time, however, the mean PLC (alpha-toxin) concentration (± SEM) detected in the contents of the birds receiving the anti- *C. perfringens* HEA feed were the same ($P = 0.57$) as that of the control feed: 51 ± 5 and 47 ± 6 mU/g contents, respectively.

Intestinal-lesion scores were significantly higher in birds receiving the anti-*C. perfringens* HEA diets than in those fed the anti-CTX HEA diets at the hour-24 and -72 sampling times (Table 5.2). The lesions commonly were observed as hemorrhagic in the ileum and caecal tonsil region.

Molecular enumeration of the hour-72 ileal samples obtained in experiment 2 revealed more *C. perfringens* in both the anti-Ctx and anti-*C. perfringens* HEA groups compared to plate counts using traditional plating on BA^{neo+} (Figure 5.4). This approach also showed no significant difference ($P < 0.05$) in *C. perfringens* abundance between treatment groups.

The *in vitro* incubation of *C. perfringens* with either anti-*C. perfringens* or anti-CTX HEA resulted in visible agglutination of *C. perfringens* in the presence of anti- *C. perfringens*, but not in the presence of anti-CTX HEA. The observed *in vitro* agglutination also resulted in a significant ($P = 0.02$) reduction in *C. perfringens* plate counts from 8.50 ± 0.06 $\log_{10}$ CFUs/mL for the samples incubated with anti-CTX HEA, to 8.29 ± 0.05 $\log_{10}$ CFUs/mL for those samples incubated with anti-*C. perfringens* HEA.

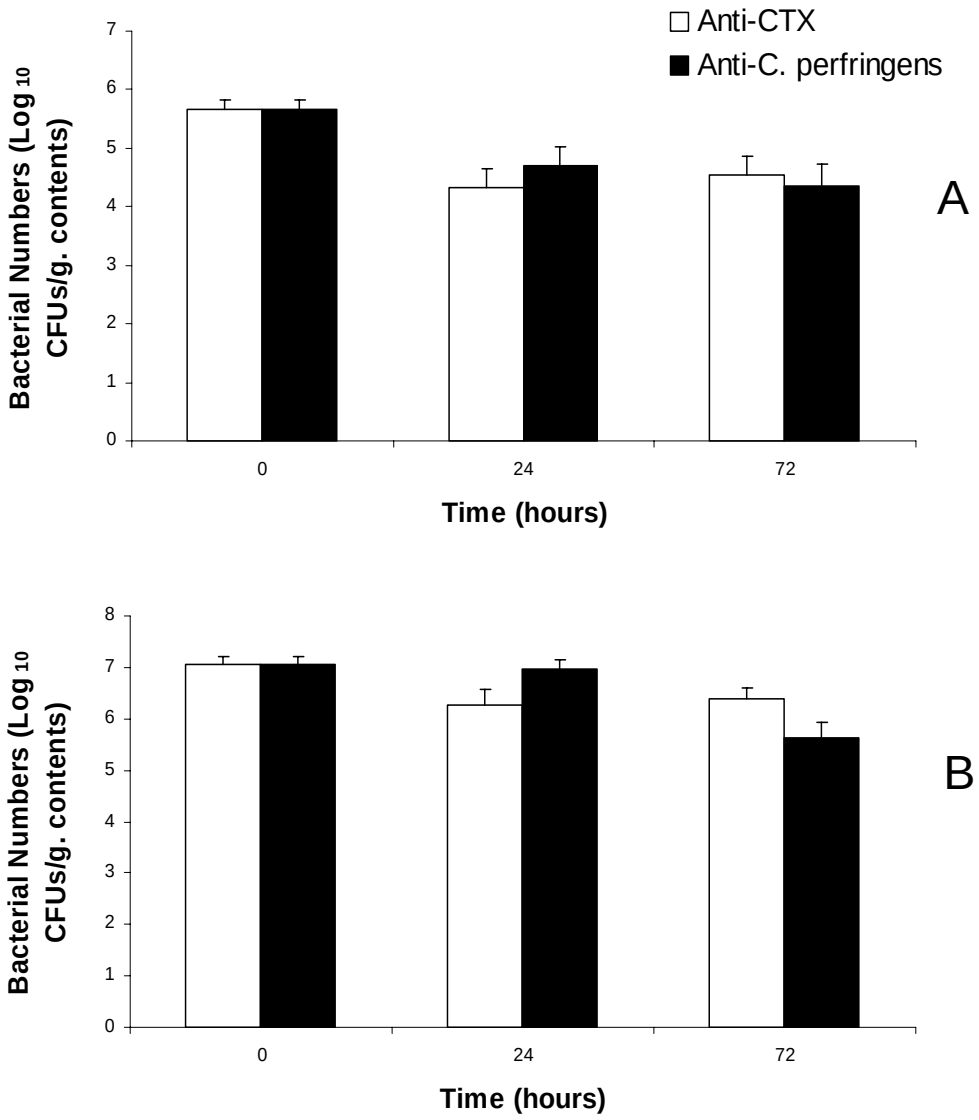

Figure 5.2. Experiment 1 ileal (A) and cecal (B) *C. perfringens* counts from challenged birds at 0, 24, and 72 hours following change to either anti- *C. perfringens* of anti-CTX HEA amended feeds. Bars represent the mean ± SEM (n = 10).

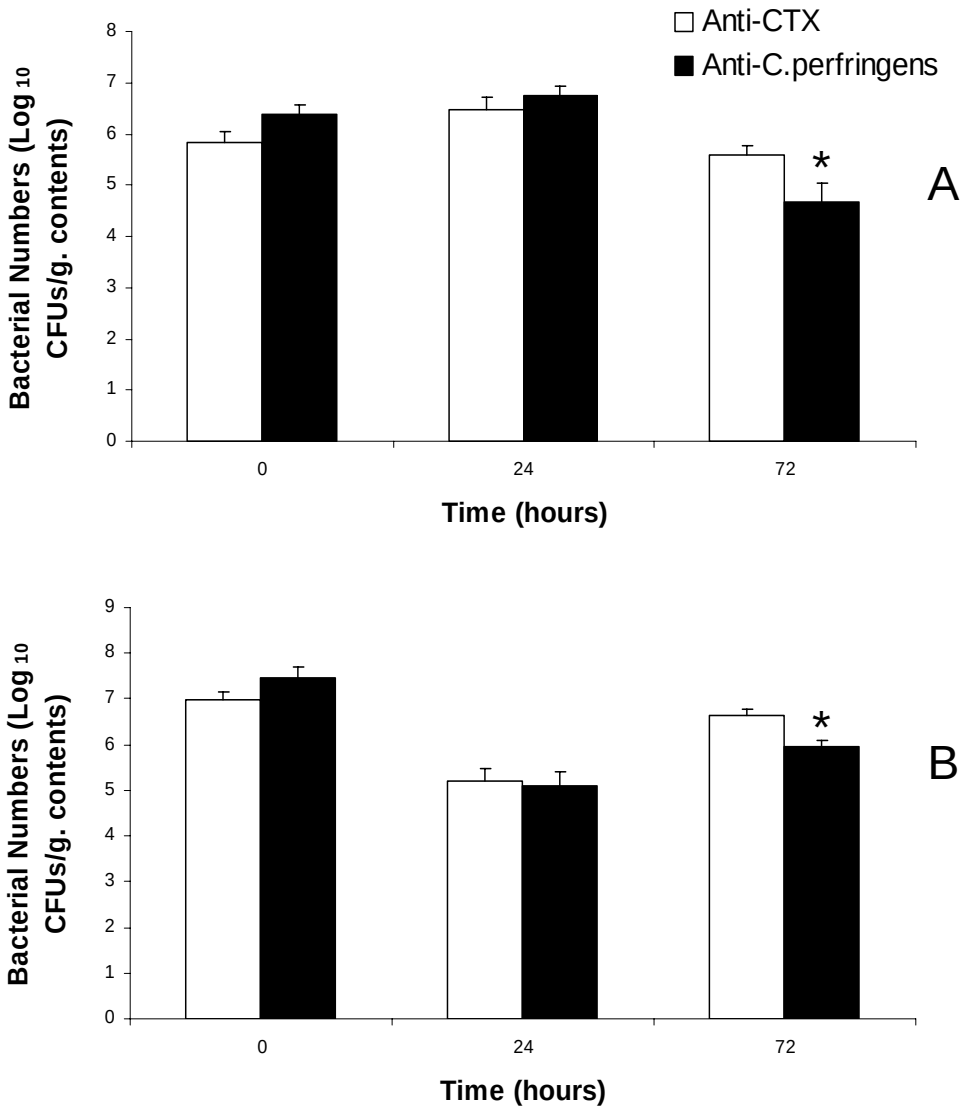

Figure 5.3. Experiment 2 ileal (A) and cecal (B) *C. perfringens* counts from challenged birds at 0, 24, and 72 hours following change to either anti- *C. perfringens* of anti-Ctx HEA amended feeds. Bars represent the mean ± SEM (n = 15). * indicates significantly lower counts ($P < 0.05$).

Table 5.2. Intestinal-lesion scores 0, 24 and 72 hours after change to antibody amended feed in Experiment 2.

	Time (h)					
	0		24		72	
Lesion Score	CTX	*C. perfringens*	CTX	*C. perfringens*	CTX	*C. perfringens*
0	5	3	5	0	1	2
1	6	1	3	3	5	0
2	2	0	2	0	1	0
3	1	1	4	6	6	3
4	1	6	1	6	2	10
n	15	11	15	15	15	15
Sum of Ranks	169	182	167	299	174	291
***P*-value**	0.09		0.01		0.02	

Values represent number of birds within a group with the lesion score indicated. The Mann-Whitney U test was used to determine differences between dietary treatment groups and the groups were considered significantly different when $P < 0.05$.

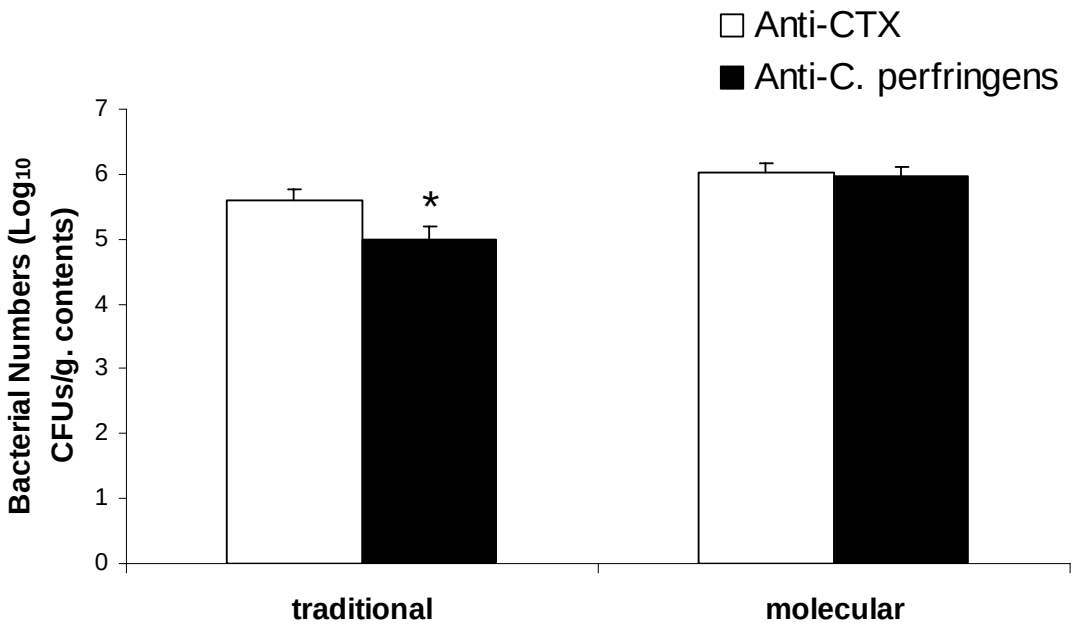

Figure 5.4. Comparison of *C. perfringens* counts in cecal contents 72 hours following change to antibody-amended feed determined by culture-based colony enumeration and real-time quantitative PCR. Bars represent the mean ± SEM (n = 15). * indicates significantly lower counts ($P < 0.05$).

5.5. Discussion

Immunization of laying hens with both the CTX and *C. perfringens* bacterin resulted in good antibody titres in the collected eggs, which after concentration and incorporation into diets, resulted in endpoint titres in excess of those used by other researchers (Yokoyama *et al.*, 1992; Yokoyama *et al.*, 1993). Using the anti-CTX amended diet, we demonstrated antibody activity throughout the entire length of the intestinal tract of broiler chickens using an ELISA antibody activity assay. We chose to use anti-CTX HEA for this because cholera toxin should not be present in the intestinal lumen of the bird and there should be no binding of the antibody to antigen that could potentially reduce the sensitivity of our ELISA. The observed activity of the administered antibody in the intestinal tract agrees with results obtained for other animal species including pigs (Yokoyama *et al.*, 1993) and rainbow trout (Lee *et al.*, 2000) where temporal and spatial HEA activity in the intestine was examined after a single bolus dose. In our trial the antibody was administered in feed and thus resulted in measurable activity throughout the entire gastrointestinal tract at 48 hours. The constant administration of antibody would be most reflective of a commercial setting where sustained presence of antibody would be necessary for prophylaxis or therapy of enteric infection. Others have attempted to improve HEA efficacy by applying treatments to protect immunoglobulins from denaturation and proteolytic degradation in the upper intestinal tract (Kovacs-Nolan and Mine, 2005; Sugita-Konishi *et al*., 2000). We used no such protection, however if done, this might have improved their efficacy.

Although only statistically significant in experiment 2, culture based enumeration showed a reduction in intestinal *C. perfringens* at 72 hours. Subjective observations

during sample collection in experiment 1 suggested lesions were more prominent in the anti-*C. perfringens* group-and in experiment 2, lesion scoring confirmed the impression. The increase in lesion score however, was not concomitant with an increase in intestinal phospholipase C activity (alpha toxin activity). Combined, these observations led us to question the validity of the observed reduction in *C. perfringens* viable counts. Enumeration of *C. perfringens* using qPCR indicated no difference in number of *C. perfringens* genomes enumerated per gram of intestinal contents. We hypothesized that the observed reduction in *C. perfringens* viable plate counts might have been due to agglutination of *C. perfringens* in the intestinal contents by the antibody. Agglutinated cells would be enumerated as a single CFU on plate counts but as multiple genomes by qPCR. In this experiment, the bacteria-antibody complexes would need to contain <10 bacteria since there was less than one order of magnitude difference in the number of *C. perfringens* CFUs between the 2 treatment groups. In support of this hypothesis, the *in vitro* agglutination of *C. perfringens* cells was readily apparent upon co-incubation with anti-*C. perfringens* antibodies, which resulted in a significant ($P = 0.02$) reduction in *C. perfringens* as measured using traditional plate counts. Several papers reported the effect of specific immunoglobulins on the *in vitro* growth of bacteria (Rainard, 1986; Reiter *et al*., 1975; Spik *et al*., 1978; Stephens *et al*., 1980). Rainard (Rainard, 1986) noted that bovine immunoglobulins induced the appearance of microcolonies in suspension due to agglutination of bacteria by the antibody. The author further stated that this could cause misleading interpretations of bacterial growth in the absence of sonic treatment. Admittedly, the conditions in the intestinal tract are more complex, involving antigen concentration and transit time, however because the molecular

enumeration of *C. perfringens* would not be affected by agglutination, this seems a plausible explanation for the observed discrepancy.

The more-severe lesions observed with anti-*C. perfringens* treatment might be related to the agglutination of *C. perfringens*. Such agglutination at the epithelial surface could be accompanied by a similar localized increase in the alpha-toxin concentration which could result in an increase in the number of lesions. Additionally, this might have involved one of the other toxins produced by *C. perfringens*, however, our *in vitro* assay examined only alpha-toxin activity. Fulton *et al*. (2002) found an increase in the infection rate among *Salmonella*-challenged birds fed anti-*Salmonella* HEA, and attributed this increased rate of infection to antibody-mediated opsonization of *Salmonella*. Perhaps opsonization of either organism or toxin might have played a role in the increased lesions, however this remains speculative.

Other researchers have shown no improvement in pathogen colonization or infection using HEA raised against bacterins (Fulton *et al.*, 2002) and also *Cryptosporidium parvum* oocysts (Kobayashi *et al.*, 2004). Kobayashi *et al.* (2004) suggested that using specific oocyst surface proteins might serve to increase the efficacy of HEA. Indeed, when antibodies are targeted to specific virulence factors such as attachment fimbria of *E. coli* (Imberechts *et al.*, 1997; Yokoyama *et al.*, 1997), or outer membrane proteins of *Salmonella* spp. (Yokoyama *et al.*, 1998a), infection reduction has been observed. These virulence factor-specific antibodies work by blocking or inhibiting attachment and thus the subsequent progression of disease (Sugita-Konishi *et al.*, 2000). It is not clear however, if the attachment of *C. perfringens* to the intestinal wall is important to the development of necrotic enteritis. What is clear is that alpha-toxin appears to be

important to the development of necrotic enteritis (Fukata *et al.*, 1988), so it seems plausible that an approach that targets this specific virulence factor might increase the potential of passive oral immunotherapy to control gastrointestinal pathogens such as *C. perfringens.*

Our results indicate that caution should be exercised when interpreting plate-count data when oral antibody approaches are used to control intestinal-pathogen colonization. Indeed, in the absence of reduced morbidity or mortality, reduced pathogen numbers alone is probably not a sufficient indicator of efficacy because agglutination of pathogen might lead to artificially low counts. In addition, although HEA have been been efficacious in controlling intestinal infection, we and others have observed increased infection when HEA are used, suggesting that this technology needs to be closely evaluated for each target pathogen and antibody specificity.

5.6. Conclusions

Our results suggest that the oral administration of hen-egg antibodies does not reduce intestinal *C. perfringens* in experimentally challenged birds. The finding of increased intestinal lesions in birds administered pathogen-specific antibodies indicate that the antibodies might in fact exacerbate necrotic enteritis. Further studies that examine antibodies that target specific virulence factors are required.

6. DIETARY AMINO ACIDS AFFECT INTESTINAL *CLOSTRIDIUM PERFRINGENS* POPULATIONS IN BROILER CHICKENS

6.1. Abstract

An experiment was performed to examine the effect of protein source and dietary amino acid profile on intestinal levels of *C. perfringens* in broiler chickens. Broiler chickens (age = 14 d; n=192) were fed diets containing 400 g/kg crude protein with fish meal, meat/bone meal, feather meal, corn gluten meal, soy protein concentrate, pea protein concentrate, or potato protein concentrate as the primary protein source along with a control diet containing 230 g/kg crude protein. The birds were orally inoculated daily, with 1 mL (~1.0 x 10^8 CFU/mL) of an overnight culture of *C. perfringens* between 14-21 days of age, killed at 28 days of age and *C. perfringens* numbers in ileum and cecum enumerated. Birds fed fish meal, meat/bone meal, feather meal and potato protein concentrate had significantly higher intestinal *C. perfringens* counts than the birds fed corn gluten meal, soy or pea protein concentrates or the control diet ($P < 0.05$). The glycine content of the diets and ileal contents was significantly, positively correlated with *C. perfringens* numbers in ileum and cecum. Dietary glycine may be an important factor in the intestinal overgrowth of *C. perfringens* in broiler chickens.

6.2. Introduction

Necrotic enteritis is a disease of broiler chickens caused by the overgrowth of

Clostridium perfringens types A and C in the small intestine (Dykstra and Reid, 1977; Fukata *et al*., 1991). Outbreaks of necrotic enteritis are sporadic and may result in high mortality and severe economic losses (van der Sluis, 2000a; 2000b). Presently, control of this disease is achieved using antibiotics and coccidiostats (Feed Additive Compendium, 2000), however, the banning of the non-therapeutic use of antibiotics in Europe, as well as consumer concerns about their use in other jurisdictions, has led to a need for a more comprehensive understanding of the factors which contribute to the development of this disease.

C. perfringens is ubiquitously distributed in the environment, and is almost always found in the intestine of healthy birds (Tschirdewahn *et al*., 1991). However, under certain conditions, *C. perfringens* proliferates rapidly resulting in the development of clinical necrotic enteritis. In spite of our present understanding of this disease, and the identification of *C. perfringens* as the etiological agent of necrotic enteritis, the predisposing factors that lead to the overgrowth of *C. perfringens* and the subsequent progression to disease are unclear. Necrotic enteritis appears to be a multi-factorial disease process in which a number of factors contribute to the development of clinical symptoms. Previous research has identified some of the predisposing factors including; management practices (Cowen *et al*., 1987; Hamdy *et al*., 1983; 1983b; Kalhusdal, *et al*., 1999), environmental conditions (Long, 1973; Kalhusdal and Skjerve, 1996), stress (Dykstra and Reid, 1977; Fukata *et al*., 1991), co-infection with *Eimeria* spp. (Al-Sheikhly and Al-Saieg, 1980), the number of *C. perfringens* present in the gut (Kalhusdal, *et al*., 1999; Williams *et al*., 2003) and diet.

Several dietary factors that have been shown to contribute to the incidence of necrotic enteritis including intestinal viscosity (Kalhusdal and Skjerve, 1996), feed processing (Branton *et al*., 1987), or diets based on wheat (Riddell and Kong, 1992) or barley (Kalhusdal and Hofshagen, 1992). In addition, it has been shown that diets with high levels of protein may also predispose birds to intestinal overgrowth by *C. perfringens* (Kalhusdal, 2000). The relationship between diet, *C. perfringens*, and necrotic enteritis is not fully understood, however, the development of nutritional strategies that reduce the intestinal numbers of *C. perfringens* may also reduce the incidence of necrotic enteritis.

Preliminary work in our laboratory examined the effect of protein source and levels on intestinal populations of *C. perfringens* (Drew *et al*., 2004). Broiler chickens were fed 230 or 400 g/kg crude protein using fish meal (FM) or soy protein concentrate (SPC) as the primary protein sources. Birds fed the FM-based diets had significantly higher numbers of *C. perfringens* in ileal and cecal digesta compared to those fed SPC-based diets at both levels of dietary crude protein. Amino acid analysis of the diets indicated that the levels of several amino acids were elevated in the FM diets compared to the SPC diets, particularly methionine and glycine. The current studies were performed to assess *C. perfringens* colonization for a wide range of protein sources and determine whether increased *C. perfringens* colonization could be correlated with the supply of specific amino acids.

6.3. Materials and Methods

6.3.1. Bacterial cultures

In all experiments a C. perfringens Type A clinical isolate from broiler chickens was

used (Dr. M. Chirino, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK). All C. perfringens cultures used for oral gavage of broiler chickens were grown anaerobically in Brain Heart Infusion (BHI) for 6 hours at 37°C (Becton, Dickinson and Co., Sparks, MD, USA) and administered when in exponential growth phase (~1.0 x 10^8 colony forming units/mL (CFU/mL)).

6.3.2. Animal management

Experimental protocols were approved by the Animal Care Committee of the University of Saskatchewan and were performed in accordance with the recommendations of the Canadian Council on Animal Care as specified in the Guide to the Care and Use of Experimental Animals (Canadian Council on Animal Care, 1993). A total of 192 (Ross 308) (Lilydale Hatchery, Wynyard, SK, Canada) were used in this experiment. To improve the intestinal colonization by *C. perfringens,* newly hatched chicks were orally gavaged in the crop with 0.5 mLs of an actively growing culture of *C. perfringens* (~1.0 x 10^8 CFU/mL) in BHI broth prepared as above using a 12.0 mL syringe equipped with vinyl tubing (I.D.0.97 mm, O.D. 1.27mm), and randomly placed into one of two battery cages. On days 1 through 14 of the experiment, birds received an unmedicated, starter diet containing 315 g/kg crude protein which met the NRC requirements for broiler chickens (NRC, 1994) (Table 6.1). On day 14, birds were wing banded, weighed, and each bird was randomly assigned to one of 16 battery cages (12 birds per cage). Two cages of birds were then randomly assigned to one of the 8 experimental diets shown in Table 6.2. A diet containing 230 g/kg crude protein and meeting NRC requirements for broiler chickens (NRC, 1994) was used as a control. The

Table 6.1. Starter diet used for all birds from days 0-14[1].

Ingredient	g/kg
Fish meal	329.4
Corn	411.6
Wheat	220.0
Limestone	16.0
Canola oil	17.0
Choline chloride	1.0
Vitamin/Mineral premix[2]	5.0

[1]Diet was formulated to contain 315 g/kg crude protein and meet NRC requirements for broiler chickens.

[2]Supplied per kilogram of diet: vitamin A, 3.3 mg; cholecalciferol, 55 μg; vitamin E, 30 mg; vitamin K, 0.5 mg; vitamin B_{12}; 0.02 mg; thiamine, 1.5 mg; riboflavin, 6 mg; folic acid, 0.6 mg; biotin, 0.15 mg; niacin, 60 mg; pyridoxine, 5 mg; 400 mg; chloride, 788 mg; sodium, 511 mg; iron, 80 mg; manganese, 21.8 mg; selenium, 0.1 mg; iodine, 0.35 mg; zinc, 100 mg.

Table 6.2. Experimental diet formulations fed to birds on days 14-28 of the experiment.

	g/kg							
Ingredient	Control	FM	MBM	FeM	PoPC	PePC	SPC	CGM
FM	169.9	569.6	-	-	-	-	-	-
MBM	-	-	646.2	-	-	-	-	-
FeM	-	-	-	422.1	-	-	-	-
PoPC	-	-	-	-	417.2	-	-	-
PePC	-	-	-	-	-	436.1	-	-
SPC	79.6	-	-	-	-	-	538.6	-
CGM	-	-	-	-	-	-	-	571.1
Corn	512.3	124.0	9.2	291.7	325.0	346.6	223.6	196.3
Wheat	200.0	200.0	200.0	200.0	200.0	200.0	200.0	200.0
Canola oil	20.0	74.2	121.5	67.8	20.0	20.0	20.0	20.0
Ca carbonate	12.1	26.2	17.0	12.3	12.9	10.1	11.8	6.7
Choline Cl	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Vit Min premix [1]	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
	1000.0	1000.0	1000.0	1000.0	1000.0	1000.0	1000.0	1000.0

[1]Same composition as Table 6.1. FM = fish meal; MBM = meat and bone meal; FeM = feather meal; PoPC = potato protein concentrate; PePC = pea protein concentrate; SPC = soy protein concentrate; CGM = corn gluten meal

other 7 diets were formulated to contain 400 g/kg crude protein using fish meal (FM), meat and bone meal (MBM), feather meal (FeM), potato protein concentrate (PoPC), pea protein concentrate (PePC), soy protein concentrate (SPC) or corn gluten meal (CGM) as the primary source of protein. All diets were isoenergetic (13.4 MJkg), did not contain antibiotics or coccidiostats and were not pelleted. Amino acid analysis of the different feeds was performed by Degussa Corporation (Allendale NJ, USA).

Birds were orally gavaged daily, from days 14 to 21 with 1.0 mL of a 6 hour broth culture of *C. perfringens* (~1.0 x 10^8 CFU/mL) in BHI, using the same method for the delivery of inoculum as above. Throughout the experimental period chicks had free access to feed and water. At 28 days of age, birds were euthanised by cervical dislocation and their intestinal tracts were removed. The contents of the ileum from Meckel's diverticulum to the ileal cecal junction and the contents of both ceca were aseptically collected from each bird with the contents from 2 birds being pooled. A subsample of the pooled ileal and cecal contents was diluted in sterile peptone containing 0.5% cysteine hydrochloride and immediately placed on ice. These subsamples were serially diluted in sterile peptone containing 0.5% cysteine hydrochloride and plated in duplicate using an automated spiral plater (Autoplate, Spiral Biotech Inc., Bethesda, MD, USA) on BBL™ Blood Agar Base (Becton, Dickinson and Co., Sparks, MD, USA) containing 5.0% sheep blood and 0.01% Neomycin Sulfate (BA^{neo+}) (The Upjohn Company, Orangeville, ON, Canada) for the enumeration of *C. perfringens*, and MRS agar (Becton, Dickinson and Co., Sparks, MD, USA) for the enumeration of lactobacilli and other lactic acid bacteria (LAB). The BA^{neo+} plates were incubated anaerobically for 24 hours at 37°C. MRS plates were incubated anaerobically

for 48 hours at 37°C. Colonies exhibiting a double zone of hemolysis on the BA^{neo+} were counted as *C. perfringens* with presumptive colonies being randomly picked, gram stained, plated on Mannitol Yolk Polymixin agar (Oxoid Inc., Napean, ON, Canada) and examined microscopically to confirm them as *C. perfringens*. The remainder of the pooled ileal contents were lyophilized and analyzed for amino acid content (Degussa Corporation, Allendale NJ, USA).

6.3.3. *In vitro* effect of various amino acids on the growth of *C. perfringens*

The growth of *C. perfringens* was assessed in an *in vitro* system using a mixed bacterial culture derived from *C. perfringens*-positive (~1.02 x 10^5CFUs/g) pooled intestinal contents from birds fed the 400 g/kg crude protein FM-based diet. In the first *in vitro* experiment, one gram of intestinal contents was resuspended in 9.0 mL of sterile minimal salts media, vigorously vortexed for 5 min and allowed to settle for 45 min. The minimal salts media consisted of equal volumes of Solution A and Solution B. Solution A consisted of (per liter) 2.0 g NaCl, 1.0 g NH_4Cl, 0.12 g $MgSO_4 \cdot 7H_2O$ and 1.0 mL of a trace element solution containing (per liter) 4.0 g EDTA, 1.5 g $CaCl_2$, 1.0 g $FeSO_4 \cdot 7H_2O$, 0.35 g $MnSO_4 \cdot 2H_2O$ and 0.5 g $NaMoO_4 \cdot 2H_2O$. Solution B consisted of (per liter) 4.24 g Na_2HPO_4 and 2.7 g KH_2PO_4. Solution A and B were autoclaved separately, cooled, and mixed aseptically. One mL of resuspended contents was then added to duplicate sterile 15 mL conical tubes containing 9.0 mL of a 10 mg/mL solution of alanine, glycine, leucine, or methionine in sterile minimal salts media. A negative control was included which contained minimal salts media alone. At time 0, 8 and 24 hours post inoculation a 50.0μL sample was removed from each conical tube and plated on BA^{neo+} plates as described above. All media was pre-reduced and the

experimental incubations were performed in an anaerobic glovebox (Forma Scientific, Marietta, OH, USA) at 37˚C. To facilitate the utilization of the added amino acids by the intestinal inoculum, and to ensure the tubes were well mixed when sampled, inoculated conical tubes were gently rocked while incubating.

In the second *in vitro* experiment, two grams of intestinal contents was resuspended in 200 mL of sterile minimal salts media, vigorously vortexed for 5 min and allowed to settle for 45 min. Ten mL of the suspension was then added to quadruplicate sterile 15 mL conical tubes containing 100 mg of either glycine, lysine, leucine, or methionine, for a final amino acid concentration of 10 mg/mL. A negative control was included which contained minimal salts media alone. At time 0, 8 and 24 hours post inoculation, a 50.0µL sample was removed from each conical tube and plated on BA^{neo+} and MRS agar plates as described above.

6.3.4. Statistical analysis

Bacterial counts and growth performance were analyzed using the general linear model of SPSS (v.10.0.5, SPSS Inc, Chicago IL, USA). Diet or amino acid treatment means were compared using the Ryan-Einot-Gabriel-Welsch multiple F test, and were considered significantly different when $P < 0.05$. Bivariate correlation analysis was performed between *C. perfringens* populations in ileum and cecum and levels of amino acids in the 8 diets and intestinal contents of the birds feed each diet. Pearson's correlation coefficient (r) was considered significant when $P < 0.05$.

6.4. Results

Subsequent to challenge with *C. perfringens* no clinical signs of necrotic enteritis were observed in any of the treatment groups or the control group. During the course of

this trial 2 birds died (one each from one of the PePC and SPC dietary groups) of causes unrelated to *C. perfringens* challenge. The ileal and cecal *C. perfringens* and LAB counts on day 28 of the experiment are shown in Figure 6.1. With the exception of chickens fed potato protein concentrate, the number of *C. perfringens* in the cecal contents of chickens fed diets that contained protein from animal sources was significantly higher than those chickens fed diets containing protein from plant sources ($P < 0.05$). Likewise, the ileal *C. perfringens* counts were found to be significantly higher in the birds fed animal protein-based diets compared to those fed the control diet or the plant protein-based diets, with the exception of the birds fed the PoPC diet which had ileal *C. perfringens* numbers that were not significantly different from either the animal protein-based diets, or the plant protein-based diets ($P > 0.05$). Cecal plate counts obtained for LAB revealed no significant differences among treatment groups, however a significantly lower number of LAB was observed in the ileum of birds fed the MBM diet compared to the group fed CGM.

The amino acid analysis of the diets and ileal contents is shown in Tables 6.3 and 6.4, respectively. Correlation analysis revealed a significant correlation ($P < 0.05$) between the level of glycine found in the diet and the number of *C. perfringens* in both ileum and cecum (Table 6.5). Correlations between all other dietary amino acids and *C. perfringens* populations were not significant ($P > 0.05$). Significant correlations between the lysine and glycine content of ileal digesta and *C. perfringens* numbers in both ileum and cecum were observed. No other ileal amino acids were significantly correlated with *C. perfringens* populations in ileum or cecum.

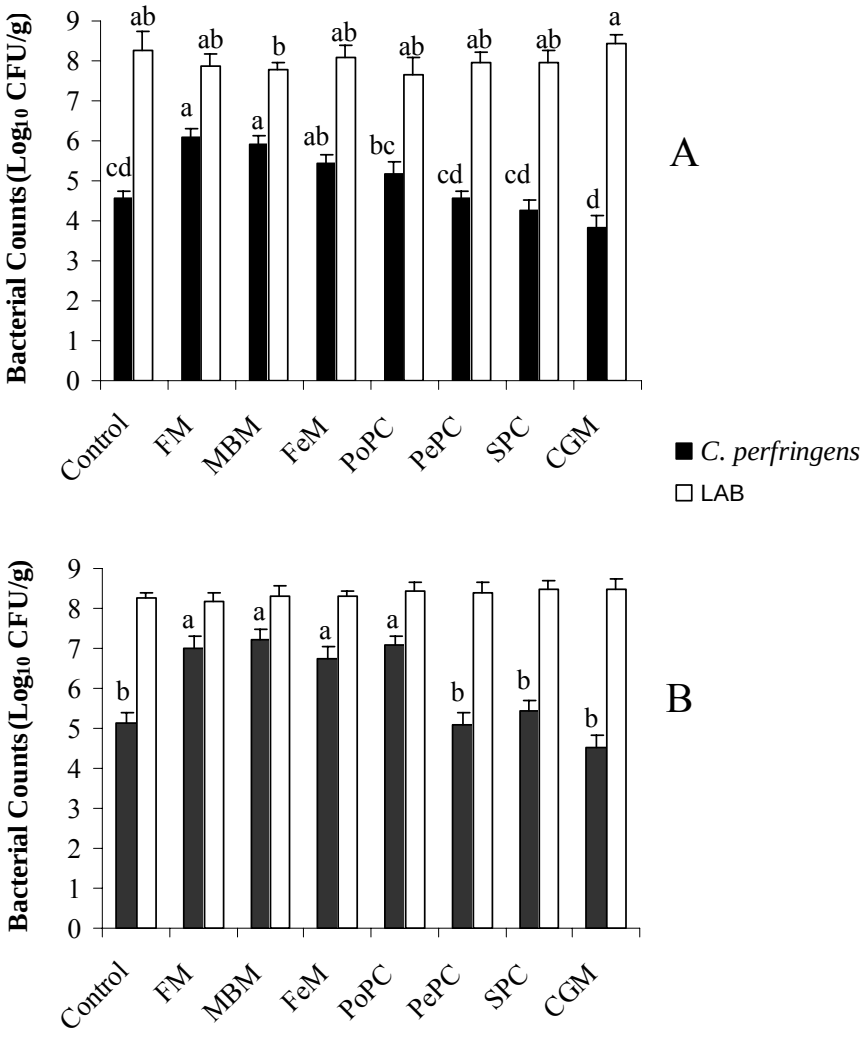

Figure 6.1. *C. perfringens* and LAB populations in ileum (A) and cecum (B) of broiler chickens fed diets based on different protein sources.. [abcd]Bars within intestinal location and bacterial species with different superscripts are significantly different ($P < 0.05$), and represent the mean ± SEM (n = 12). FM = fish meal; MBM = meat and bone meal; FeM = feather meal; PoPC = potato protein concentrate; PePC = pea protein concentrate; SPC = soy protein concentrate; CGM = corn gluten meal

Table 6.3. Protein and amino acid composition of the diets (as is basis).

	g/kg							
	Control	FM	MBM	FeM	PoPC	PePC	SPC	CGM
Protein (N x 6.25)	231.5	400.9	413.3	409.1	341.8	469.6	402.3	363.1
Met	4.7	10.1	5.5	3.4	7.9	4.2	5.2	8.7
Cys	3.1	3.8	4.1	16.4	5.3	4.8	5.6	7.0
Lys	13.1	25.2	19.7	10.9	25.9	29.3	21.6	6.5
Thr	8.6	16.0	13.1	19.4	19.7	15.1	14.4	13.4
Trp	2.6	3.6	nd	2.9	4.6	3.3	4.8	2.2
Arg	13.3	26.2	26.5	29.2	19.5	34.3	26.6	12.8
Ile	9.5	14.2	11.2	19.7	20.1	20.3	17.2	15.9
Leu	19.2	27.7	24.5	36.7	38.1	36.9	30.2	64.7
Val	11.2	17.4	16.5	29.7	24.0	22.0	18.4	18.7
His	7.2	9.9	8.5	6.4	9.4	11.0	10.7	8.7
Phe	10.1	14.6	13.2	20.8	22.5	23.1	18.5	24.3
Gly	11.4	36.7	47.9	33.4	18.6	17.7	16.0	11.5
Ser	9.7	18.0	16.5	44.3	19.6	21.2	18.5	21.1
Pro	13.9	24.3	31.8	41.4	21.0	21.5	22.3	38.3
Ala	13.3	27.2	27.4	21.9	20.2	19.4	17.3	35.4
Asp	19.8	34.0	29.3	29.7	42.8	47.8	40.4	24.1
Glu	39.2	56.5	54.5	56.1	49.1	76.7	72.9	91.0

FM = fish meal; MBM = meat and bone meal; FeM = feather meal; PoPC = potato protein concentrate; PePC = pea protein concentrate; SPC = soy protein concentrate; CGM = corn gluten meal

Table 6.4. Amino acid content of ileal contents (dry matter basis).

	g/kg							
	Control	FM	MBM	FeM	PoPC	PePC	SPC	CGM
Protein (N x 6.25)	175.3	255.5	300.0	488.8	246.0	245.1	172.7	226.7
Met	2.8	4.9	3.1	3.5	4.2	3.6	1.8	3.4
Cys	3.5	4.1	5.0	26.4	7.5	6.2	3.9	6.5
Lys	8.4	12.9	12.2	14.1	13.6	12.2	6.8	5.6
Thr	9.2	11.5	10.2	25.3	16.4	11.8	9.3	10.7
Trp	2.3	3.4	2.4	4.2	4.9	3.4	2.1	2.4
Arg	6.5	11.3	14.1	24.3	9.5	11.9	5.9	6.9
Ile	6.7	9.0	7.2	17.1	12.6	11.7	6.3	8.9
Leu	11.3	14.3	14.2	34.3	19.4	18.4	10.2	23.0
Val	8.4	10.9	10.4	28.4	14.9	14.3	7.6	11.3
His	4.6	6.0	5.4	7.3	6.0	5.5	3.9	5.7
Phe	6.9	9.3	7.9	17.8	12.1	11.6	6.1	10.4
Gly	10.6	21.2	32.6	35.5	14.2	12.5	9.0	11.0
Ser	8.3	11.7	11.9	46.1	14.5	11.9	7.6	11.4
Pro	9.2	12.0	20.2	47.0	11.6	11.3	7.4	17.9
Ala	9.1	13.2	16.1	22.0	12.3	12.3	7.6	14.6
Asp	22.2	34.4	29.9	52.8	29.2	25.2	26.4	16.8
Glu	19.4	28.4	30.6	59.2	28.3	25.7	21.7	37.3

FM = fish meal; MBM = meat and bone meal; FeM = feather meal; PoPC = potato protein concentrate; PePC = pea protein concentrate; SPC = soy protein concentrate; CGM = corn gluten meal

Table 6.5. Pearson correlation (r) coefficients between the amino acid content of diets and ileal digesta and intestinal populations of *C. perfringens*.

	Dietary Amino Acids		Ileal Amino Acids	
	Ileum	Cecum	Ileum	Cecum
Crude Protein	0.222	0.181	0.496	0.501
Met	0.152	0.152	0.561	0.466
Cys	0.058	0.144	0.203	0.267
Lys	0.343	0.347	0.809*	0.801*
Thr	0.360	0.555	0.312	0.425
Trp	-0.252	-0.095	0.396	0.536
Arg	0.408	0.339	0.565	0.533
Ile	-0.162	0.019	0.228	0.306
Leu	-0.525	-0.426	0.066	0.127
Val	0.154	0.316	0.272	0.327
His	-0.146	-0.132	0.482	0.481
Phe	-0.422	-0.250	0.206	0.263
Gly	0.893*	0.807*	0.759*	0.714*
Ser	0.168	0.236	0.302	0.346
Pro	0.105	0.136	0.305	0.319
Ala	0.083	0.041	0.449	0.429
Asp	0.044	0.157	0.631	0.648
Glu	-0.588	-0.589	0.227	0.266

*Correlation coefficients are significant ($P < 0.05$)

The results from the first *in vitro* experiment are shown in Figure 6.2. Although no significant differences were observed at 0 or 8 hours, at 24 hours the number of *C. perfringens* was significantly increased in medium containing glycine compared to the control medium (5.33 ± 0.01 and 5.10 ± 0.03 $\log_{10}$ CFU/mL, respectively). In contrast, media containing methionine, leucine or alanine supported significantly lower numbers of *C. perfringens* compared to the control (4.87 ± 0.03, 4.98 ± 0.04, and 5.01 ± 0.04 $\log_{10}$ CFU/mL, respectively).

In the second *in vitro* experiment (Figure 6.3), similar results were observed with no significant differences observed at hours 0 or 8. At 24 hours, however, significantly higher numbers of *C. perfringens* were observed in both the glycine and lysine amended cultures (5.86 ± 0.03 and 5.66 ± 0.07 $\log_{10}$ CFU/mL, respectively), compared to the methionine, leucine, or the negative control (4.88 ± 0.07, 4.90 ± 0.05 and 4.77 ± 0.04 $\log_{10}$ CFU/mL, respectively). In addition, significant differences were observed in the ability of the various amino acids to support the population of LAB from the same intestinal contents. After 24 hours incubation, the glycine-containing media supported significantly fewer LAB than the lysine or methionine containing media (7.26 ± 0.02, 7.82 ± 0.03 and 7.70 ± 0.06 $\log_{10}$ CFU/mL, respectively).

6.5. Discussion

Although necrotic enteritis is an economically important disease of broiler chickens, the development of a reproducible experimental model for this disease has proven difficult. *C. perfringens* is recognized as the etiological agent of necrotic enteritis but other contributing factors are usually required to initiate clinical disease. These predisposing factors include management stress, sub-clinical intestinal coccidiosis,

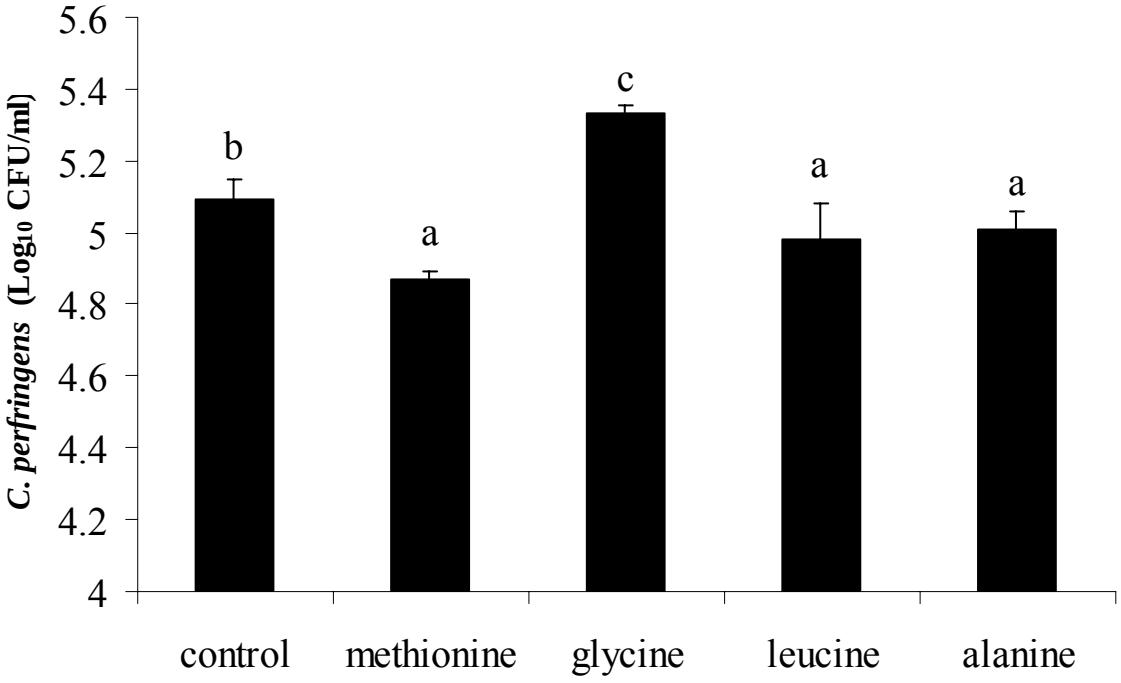

Figure 6.2. *In vitro* growth of *C. perfringens* after 24 hours incubation of intestinal contents in minimal salts media supplemented with different amino acids[1].

Footnotes to Figure 2.

[abc]Bars with different superscripts are significantly different ($P < 0.05$), and represent the mean ± SEM (n = 4). Final concentration of amino acids was 10 mg/mL

[1]Mean *C. perfringens* count at time 0 hours was 5.09 Log_{10} CFU per mL of culture (SEM = 0.05).

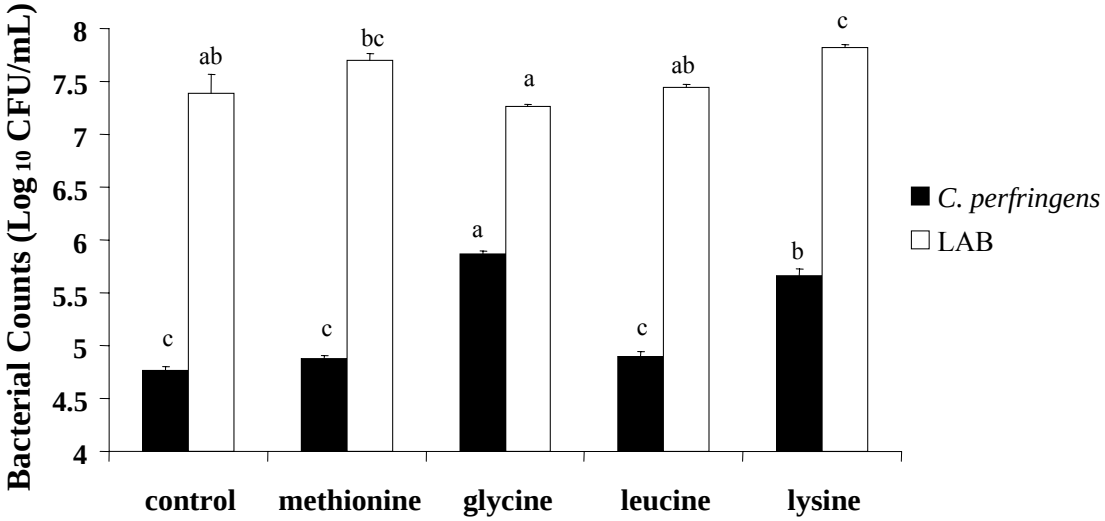

Figure 6.3. *In vitro* growth of *C. perfringens* and LAB after 24 hours incubation of intestinal contents in minimal salts media supplemented with different amino acids[1].

Footnotes to Figure 3.

[abc]Bars with different superscripts are significantly different ($P < 0.05$), and represent the mean ± SEM (n = 4). Final concentration of amino acids was 10 mg/mL

[1]Mean of *C. perfringens* and LAB numbers per mL of culture material at time 0 hours was 4.93 Log_{10} CFU/mL (SEM = 0.07) and 5.48 Log_{10} CFU/mL (SEM = 0.02), respectively.

changes in dietary formulation and alteration of feeding programs (Kalhusdal, 2000; Shane *et al*., 1985). These factors are numerous, but many are ill-defined and experimental results have been contradictory (Kalhusdal *et al*., 1999). This lack of a reliable experimental model for necrotic enteritis has hampered efforts to evaluate putative risk factors and preventive measures. The model used in the current study is based on populations of *C. perfringens* in the ileum and cecum and no overt clinical signs of necrotic enteritis were noted during the experiment. The numbers of *C. perfringens* in the normal chicken gut vary considerably from sporadic and low numbers to 10^7 CFU/g or more (Ficken and Wages, 1997; Engberg *et al*., 2002). However, since *C. perfringens* is the etiological agent of necrotic enteritis it seems reasonable to assume that increased numbers of this organism in the gut are associated with an increased predisposition to clinical necrotic enteritis.

Dietary composition has been shown to have important effects on the incidence of necrotic enteritis in broiler chickens. Long and Truscott (1976) used a pure culture challenge model to reproduce necrotic enteritis in broiler chickens with total necrotic enteritis mortality ranging from 1-28%. This model was later modified to feed birds elevated dietary concentration of fish meal prior to initiation of inoculum administration and intra-duodenal administration of inoculum (Truscott and Al-Sheikhly, 1977). Elevated concentration of dietary wheat and barley (Riddell and Kong, 1992), animal proteins (Kalhusdal and Skjerve, 1996) and zinc (Baba *et al*., 1992) may also exacerbate necrotic enteritis. More recently, Williams *et al*. (2003) reported that a combination of diet (high inclusion of wheat and fish meal), infection with *Eimeria maxima* followed by per cloaca inoculation with *C. perfringens* 6 days later resulted in intestinal lesions

but no mortality. Clearly, diet has a significant impact on *C. perfringens* populations in the intestinal tract but the causative factors associated with various diets have not been identified.

Kaldhusdal and Skjerve (1996) performed a longitudinal study of necrotic enteritis and broiler chicken feeding practices in Norway over a 20 year period. They reported that the increased use of rendered animal products was associated with an increase in the incidence of necrotic enteritis. Several experimental studies have reported that the source and level of protein in diets significantly affects the number of *C. perfringens* in intestinal contents in chickens (Drew *et al*., 2004), pigs (Mansson and Smith, 1962; Mansson and Olhagen, 1967) and dogs (Zentek, 2000; Zentek *et al*., 1998; 2003). Studies were performed in the 1960s in pigs on the effect of dietary protein level on the numbers of *C. perfringens* shed in feces. Mansson and Smith (1962) placed 8-week-old pigs on a diet high in crude protein and reported that there was an increase in the number of *C. perfringens* type A from about $2.5x10^2$ per gram in feces from pigs on a normal diet to 10^6 per gram in the feces of pigs on the high protein diet. In another study, Mansson and Olhagen (1967) demonstrated that when pigs were fed on a high protein diet the number of *C. perfringens* type A was significantly increased in the feces 5 to 10 days after being put on the diet.

Zentek (2000) compared the effect of feeding a low protein diet (22% crude protein) to a high protein diet (63% crude protein) containing 51% greaves meal (beef/pork by-product) to dogs. Intestinal populations of both *Streptococcus* spp. and LAB were unaffected by diet, but intestinal numbers of *C. perfringens* increased from 10^6 to 10^9 CFU/g. In another study, Zentek *et al*. (1998) compared the effect of feeding high

protein diets composed of either protein from animal (greaves meal) or plant (soy protein hydrolysate or corn gluten meal) sources. Dogs fed greaves meal diets had increased levels of *C. perfringens* in their ileal chime, compared to animals fed the plant-based protein diets.

A previous study in broiler chickens examined the effects of protein source (animal vs. plant protein) and protein concentration on intestinal populations of *C. perfringens* in broiler chickens (Drew *et al*., 2004). Fish meal and soy protein concentrate were the animal and plant protein sources used at crude protein levels ranging from 230 to 400 g/kg. At 28 days of age, *C. perfringens* counts in ileum and cecum increased significantly ($P < 0.05$) as crude protein level increased from 230 to 400 g/kg in birds fed fish meal-based diets but not in birds fed SPC-based diets. Thus, crude protein level alone does not adequately explain the changes in *C. perfringens* populations.

In the present study, *C. perfringens* numbers were elevated in chickens fed animal protein-based diets (FM, MBM and FeM) compared to those fed plant protein-based diets (PePC, SPC, CGM). However, PoPC did not fit this pattern and numbers of *C. perfringens* were similar to those seen in the birds fed animal protein-based diets. Correlation analysis of the amino acid composition of the diets and *C. perfringens* populations showed that only glycine was significantly correlated with *C. perfringens* numbers. The glycine content of the animal protein-based diets was approximately 2 to 4 times that found in plant protein-based diets. Potato protein concentrate had the highest glycine content of the plant-based ingredients (18.6 g/kg) and this may have accounted for the high *C. perfringens* seen in birds fed the diet based on this protein. In ileal contents, glycine and lysine content was significantly correlated with *C.*

perfringens numbers. While interesting that a correlation for glycine and lysine was observed in the ileal contents, the amino acid content of the ileal contents reflects unavailable dietary protein, endogenous protein losses, and the net effect of bacterial nitrogen metabolism. Thus, ileal glycine content is not necessarily a reflection of dietary glycine content complicating the interpretation of this correlation.

Although populations of *C. perfringens* were affected significantly by diet, with the exception of a significant increase in LAB populations in the CGM-fed birds compared to the group fed MBM, there was no effect of diet on LAB numbers in ileum or cecum. Zentek *et al*., (1998) also reported that the number of LAB and *Streptococcus* spp. in the intestinal contents of dogs were unaffected by the level or source of protein. However, a later study (Zentek *et al*., 2003) reported that the number of bifidobacteria were reduced to undetectable levels in the feces of dogs fed diets containing 726 g/kg of crude protein while populations of *C. perfringens* were significantly increased. Thus it is unclear whether the diets employed in the current study influenced *C. perfringens* colonization in the intestine directly or indirectly by modifying colonization of commensal bacteria other than LAB.

Results of the *C. perfringens* challenge study suggested dietary constituents common among animal source proteins and potato meal support increased *C. perfringens* colonization of the intestine, or alternatively, plant based protein sources contain constituents which inhibit *C. perfringens* colonization. Since glycine content was markedly higher in the diets supporting *C. perfringens* colonization and glycine content positively correlated with C. *perfringens* counts, we tested the effect of various amino acids on *C. perfringens* growth *in vitro*.

Although an *in vitro* approach using a *C. perfringens* pure culture would yield results demonstrating the ability of *C. perfringens* to use various amino acids, it fails to take into account competition for the amino acids from other community members or co-metabolic processes which both may influence the actual proliferation of *C. perfringens in vivo*. Thus, a mixed culture was used which was obtained from intestinal contents of birds which had received the FM diet in the feeding experiment.

For both *in vitro* trials glycine was shown to support significantly higher growth of *C. perfringens* in mixed culture after 24 h. In the second trial lysine was shown to also be capable of supporting *C. perfringens* in mixed culture supporting the observation that the lysine content of ileal digesta was significantly correlated with *C. perfringens* populations in ileal and cecal contents. In contrast, glycine supported significantly less growth of LAB compared to lysine. These results were contradictory to the results we obtained *in vivo* where no significant effect of glycine on LAB colonization was observed. In the absence of additional nutrients, the reduction in the available nutrient pool may have resulted in exaggerated differences in various community members.

The present studies support the hypothesis that the dietary glycine may play an important role in supporting the overgrowth of *C. perfringens*, in the ileum and cecum of broiler chickens thereby increasing the potential for an outbreak of clinical necrotic enteritis. This is further supported by the observation that glycine is stimulatory for the both growth of *C. perfringens* and alpha toxin production (Ispolatovskaya, 1971; Nakamura *et al*., 1978). Improved knowledge of the effect of specific dietary components on intestinal *C. perfringens* populations may aid in the formulation of

broiler diets that reduce the incidence of necrotic enteritis especially in the absence of growth promoting antibiotics.

7. OVERALL CONCLUSIONS

In addition to anecdotal evidence, there is a considerable body of evidence in the literature which would suggest that the administration of probiotics, and in particular organisms such as *Bifidobacterium* and *Lactobacillus* spp., does improve animal health. Proposed mechanisms include competition for both intestinal binding sites, and a limited nutrient supply. It seemed logical therefore to expect that constant administration of a probiotic organism of chicken origin would result in a reduction in the number of allochthonous microorganisms, including human pathogens such as *S. enteritidis* and *C. jejuni*. In terms of these two model pathogens however, we did not observe any significant differences between our treatment groups and negative controls. This despite the fact that both probiotic organisms used were of chicken origin and their ability *in vitro* to survive the harsh conditions encountered in the proximal intestine. Moreover, in the case of *L. fermentum*, our isolate performed better than commercial Lactobacillus cultures sold as probiotics. The results suggest that in addition to being able to withstand the first line of intestinal defence such as the bile and low pH encountered in the crop, stomach, and proximal small intestine, other factors clearly contribute to a probiotic microorganism's ability to out compete allochthonous microorganisms, and pathogenic microorganisms in particular. These factors may include the prerequisite production of antimicrobial compounds by potential probiotic strains, or more poorly defined factors such as the ability to specifically modulate the

chicken immune system. Thus, there is a need to further investigate, and ultimately identify, all the factors that contribute to the success or failure of a probiotic microorganism if they are to receive widespread use and acceptance.

Passive oral immunization of other animal species with hen-egg antibodies has proven to be very efficacious in reducing the morbidity and mortality caused by enteric diseases of bacterial origin. In our studies however, using our challenge model we were unable to demonstrate their ability to reduce the level of intestinal colonization of our model pathogens. These negative results were observed despite their demonstrated survival and activity in the entire gastrointestinal tract, as well as their *in vitro* ability to reduce intestinal or mucus attachment by *S. enteritidis* or *C. jejuni*, respectively. This may have been due, in part, to the fact that *S. enteritidis* and *C. jejuni* are not poultry pathogens. Because poultry merely serve as a reservoir for these human pathogens, these two microorganisms would lack the appropriate virulence factors, such as attachment pili, which would stimulate and subsequently be used by the host to clear the infection. Even if our antibodies were specifically targeted to the virulence factors which make them pathogenic to humans, it is uncertain whether they would make a difference in the chicken model. With the poultry pathogen *C. perfringens*, attachment does not appear to be a prerequisite for infection and thus there was no significant reduction in the level of intestinal colonization when hen-egg antibodies were administered. Surprisingly, however, there was an increase in the frequency and severity of intestinal lesions. Although the reasons for this are unclear, the lesions seemed most severe in the cecal tonsil region and as such it may be due to opsonization of α–toxin, the microorganism itself, or both. Although this approach may hold

promise, in terms of our three model pathogens, there is a disconnect between functionality and efficacy, and more research is required to identify the specific virulence factors required by pathogens to cause disease and if these are appropriate for the animal model in question.

Identifying pathogen-specific virulence factors may ultimately increase the efficacy of intervention strategies such as hen-egg antibodies, however the identification of predisposing factors, which can actually increase the risk of disease in the first place, may be of equal or more importance. Identification of predisposing factors might allow us to better manage the microbial ecology of the gut and is illustrated by our study examining the relationship between dietary protein and necrotic enteritis. Using diets that varied in the protein source used for formulation, it was shown that dietary protein, and amino acid profile in particular, may play a role in predisposing broiler chickens to necrotic enteritidis by specifically altering intestinal bacterial populations. Thus, it seems logical that dietary manipulation may have a role that is equally important in disease prevention than any intervention strategy. Complicating the relationship that diet may have on managing the microbial ecology of the gastrointestinal tract is the observation that a large number of dietary components appear to influence the intestinal microflora. Clearly, more research is needed if we are to identify which dietary factors are involved in each particular disease, and if the nature of the observed effect is indirect or direct.

In the current studies, a direct challenge model was used for the all three pathogens. In all three challenge models birds were orally gavaged with a consistent titre of pathogen. To what extend this model reflects the encounter of pathogen in a commercial

setting is questionable. For both *S. enteritidis* and *C. jejuni*, a review of the literature indicated that a challenge dose of 1 x 10^5 CFU/mL or less resulted in inconsistently colonized birds. In all likelihood even this challenge dose is artificially high relative to the challenge dose experienced in commercial poultry flocks. However, in an attempt to ensure a consistent and uniform level of colonization from an experimental point of view, we chose to orally gavage birds with 1 x 10^8 CFU/mL. In this respect, our challenge model was effective, however in the end the two intervention strategies were unsuccessful. It is possible that the high challenge dose overwhelmed the intervention strategies investigated and that under lower challenge doses, more representative of a commercial setting, a better outcome may have been observed. Employing a "Trojan" or seeder-model may have been a more realistic approach to mimic pathogen challenge in a commercial setting. Not only would this approach better model the natural spread of pathogens within poultry flocks, but it might be a better model for pathogen control using biological measures such as hen-egg antibodies or probiotics.

In addition to the high pathogen titres used to infect birds, timing of the pathogen challenge may have had a negative effect on the experimental outcome of all trials. The ability of the probiotics or egg-antibodies to reduce or exclude pathogens may have been increased had they been administered prior to the administration of the pathogens. With probiotics in particular, niche occupation by *L. fermentum* or *B. animalis* may have excluded, or reduced, the ability of the pathogens to become established in the gastrointestinal tract. In particular, this may have been beneficial with *S. enteritidis* where epithelial attachment and translocation may be more important in the persistence of *S. enteritidis* in poultry flocks, however a mucus layer covered by antibody or

probiotic may also inhibit intestinal colonization by *C. jejuni*. In retrospect this seems logical, as niche occupation by *C. perfringens* was the rationale for gavaging the birds on day-of-hatch and was very effective in ensuring intestinal colonization. If the timing of the pathogen challenge was changed to post probiotic or egg-antibody administration, perhaps revisiting the model would have been appropriate as well.

The challenge model used for *C. perfringens* was different and in part reflected the unique nature of an opportunistic pathogen. Not only were birds orally gavaged with a high challenge dose on day-of-hatch, but they were orally gavaged for an additional seven days from day 14 through 21. This challenge model was based on work done by both others and ourselves and resulted in a very reliable model for intestinal colonization. This challenge model however, did not produce clinical necrotic enteritis. Nearly without exception, all current necrotic enteritis models employ a polymicrobial challenge using *Eimeria* spp., yet the etiological agent is the common intestinal inhabitant *C. perfringens*. We chose to examine intervention strategies and dietary factors that contribute to intestinal colonization by *C. perfringens* in the absence of disease, rather than attempting to address the more complex factors that control a polymicrobial infection.

Despite the intestinal colonization levels that we were able to achieve using our challenge models, some birds appeared to be pathogen free at the time of sampling while others were highly colonized. This illustrates the weakness of not only our challenge model, but also all current experimental models for these three pathogens. Our limited understanding of the extremely complex microbial ecology of the gastrointestinal environment makes model design crude at best and more often than not

flawed. Proper experimental models require an increased understanding of the host, the microbes, the relationship between intestinal microbes and the host, as well as factors that control microbial community dynamics within the gastrointestinal tract. Presently this area of research is in its' infancy, however we are making steps towards a more complete holistic understanding of these complex relationships.

In conclusion, although the approaches used in these studies may show promise for the reduction of pathogens in broiler chickens, a more thorough and comprehensive understanding of the factors that help or hinder their efficacy is required. For oral administration of antibodies to be effective, the identification of the most appropriate virulence factors that should be used to immunize hens is essential. In instances when targeting a bacterial pathogen which is not normally a pathogen in the broiler chicken, as was the case with *S. enteritidis* and *C. jejuni*, this approach may not be useful. With a chicken pathogen such as *C. perfringens* however, this approach might be useful, and our results suggest that identification and use of the correct target antigen is crucial. In this respect, the most appropriate direction for further research may be assessing the usefulness of raising HEA specific to the α-toxin. In addition, our results support the notion that diet may be our most effective way to manage the microbial ecology in the absence of medicated feed. At the very least, understanding the dietary factors which predispose birds to specific diseases such as necrotic enteritis will go a long way to fighting disease in an antibiotic-free agricultural sector. In the end it may be that, due to the biological nature of these approaches, they may never be as effective as antibiotics; however they will have to deliver consistent and reliable results if they are to receive widespread acceptance and use.

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9. APPENDICES

9.1. Appendix A

Supplemental images for Chapter 5.

To evaluate HEA-mediated agglutination, 3.3 X 10^8 CFUs/mL *C. perfringens* were incubated with anti- *C. perfringens* and anti-CTX HEA at an endpoint tire of 1:72,000 for 15 min at room temperature. The incubated solutions were placed on glass slides and agglutination visually evaluated under 400X magnification on a Axiostar *plus* Zeiss microscope (Carl Zeiss Vision GmbH, Germany) fitted with Axiocam MRc camera. The resulting images are shown in Figure A.1

Figure A.2 is a photomicrograph showing typical lesions found in the cecal tonsil region of birds challenged with *C. perfringens* in Chapter 5. Images are of hemotoxylin- and eosin- stained, formalin-fixed tissue using a Axiostar *plus* Zeiss microscope (Carl Zeiss Vision GmbH, Germany) fitted with Axiocam MRc camera.

Figure A.1. Photograph taken under phase contrast (400X) showing the *in vitro* agglutination of *C. perfringens* by anti-Ctx HEA (A) compared to that of anti- *C. perfringens* HEA (B). The antibodies were diluted 1:72,000 and incubated in the presence of 8.50 $\log_{10}$ CFUs *C. perfringens.*

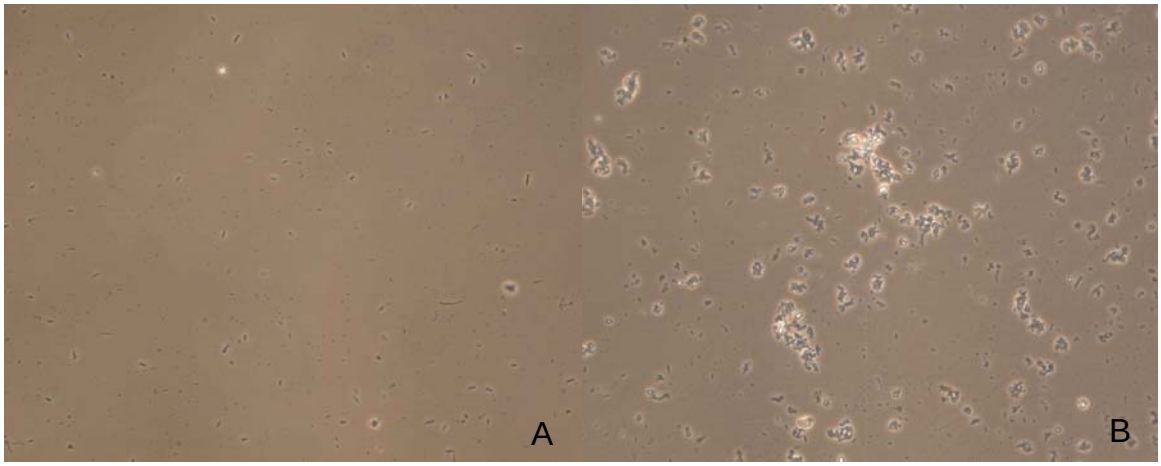

Figure A.2. Micrograph of H&E stained cecal tonsil cross sections at 50X (A) and 400X (B). Hemorrhagic lesions are evident by the infiltration of erythrocytes at the villus tips.